TWENTY-SIX AFTERNOONS OF BIOLOGY

An Introductory Laboratory Manual

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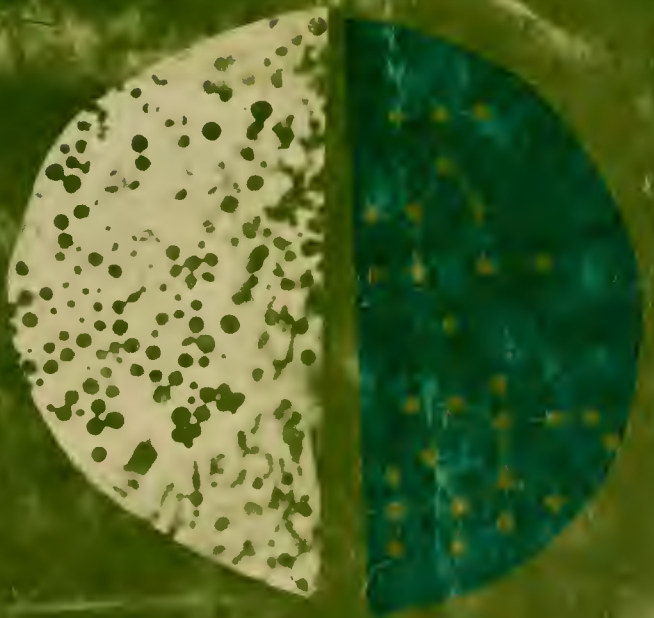

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Presented by

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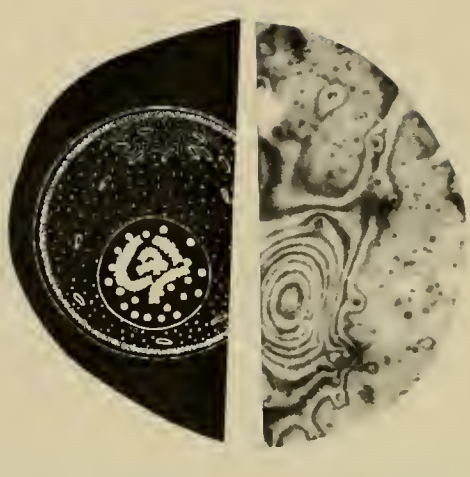

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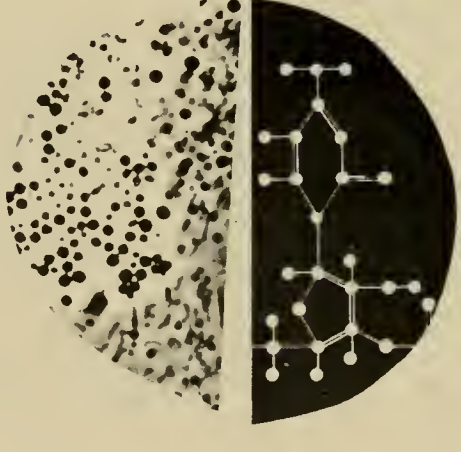

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TWENTY-SIX AFTERNOONS OF

BIOLOGY

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ADDISON-WESLEY PUBLISHING COMPANY, INC.
READING, MASSACHUSETTS · PALO ALTO · LONDON

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PREFACE

The introductory biology course for which this book is the laboratory manual comes in a period of extraordinary changes. On the one hand, we are undergoing a revolution in biology, which for the first time is approaching its problems systematically at the molecular level. With the emergence of biology at this level, already occupied by chemistry and physics, science as a whole has achieved a new unity.

At the same time we are undergoing a fundamental revolution in American education. Its main seat is not in the colleges, but the high schools. Exhortations, threats, normal internal developments, improved economic conditions, federal programs for retraining teachers, science fairs and competitions, and probably most important of all, the advanced placement program—all these have had their effect, and students now enter college in a very different condition from what obtained just a few years ago. Many of them know much more science and mathematics than they ever did before. Indeed, many of them have gone far past what we taught juniors and seniors in the colleges only a few years ago.

What is much more important is that high-school students quite generally have developed a new eagerness to learn and understand science. The glamor that used to go with athletic achievement seems largely now to be accorded scientific achievement. The elation that students used to derive from working their muscles, many now seem to achieve also by working their heads. Surprisingly learned, eager, responsive, deeply interested—this is the new college freshman. This book is dedicated to him.

The course for which this book is the laboratory guide has been given on a reasonably large scale to approximately 350 students, mainly freshmen and sophomores, about evenly divided between general education students and those intending to concentrate in the sciences, mainly premedical students. No distinction whatever is made in handling these two groups together; and it is noteworthy that after a short initial lag, the general education students keep up thoroughly with the others.

Each student has one three-hour laboratory session weekly throughout two semesters. At Harvard this comes out to thirteen sessions per semester. Each laboratory section contains about twenty-five students, supervised by two graduate student assistants, and under the general supervision of one of the senior staff who is continuously available. In twenty-six laboratory sessions we do everything described in this book. It makes a keyed-up, busy laboratory, yet not a harrassed one. If at any point we thought the work of the laboratory was becoming too pressed for time, we would cut down on its content.

It may help place the laboratory work in perspective to know something of its relation to the lectures in our course. We have three one-hour lectures per week through two semesters. No attempt is made to synchronize the laboratory work with the lectures; each attempts to develop its own logic. Nevertheless numerous points of correspondence and overlap develop between these two aspects of the course, and by its end lectures and laboratory tend to form a reasonably unified whole. Some idea of the content and sequence of the lectures can be gained from the outline of lecture topics that follows.

I. Origin of life (2 lectures)

II. Ultimate particles
   1. Interconversion of matter and energy
   2. Structure of the atomic nucleus
   3. Nuclear transformations: origin of sunlight

III. Structure of the atom
   1. Atomic orbitals; inert gases
   2. Periodic system of the elements

IV. Chemical combination
   1. Ion formation
   2. Molecule formation: the covalent bond
   3. Coordinate valence (the dative bond)
   4. Hydrogen bonds
   5. Van der Waals forces
   6. Polar molecules: surface forces, the association of water

V. Organic molecules
   1. Special position of C, H, N, O
   2. The major groups (hydroxyl, carbonyl, carboxyl, amino, sulfhydryl, etc.)

VI. Biomolecules
   1. Sugars, disaccharides, polysaccharides
   2. Neutral fats; phospholipids
   3. Amino acids
   4. Nucleotides

(This entire treatment of molecules, beginning with the discussion of chemical combination, is "morphological." It is conducted entirely in terms of structural formulas. There is rarely an empirical formula in our discussions. The construction of three-dimensional models of the molecules in the laboratory is an important element in this instruction.)

VII. "The alphabet of organisms"
   1. Four ultimate particles: protons, neutrons, electrons, photons
   2. Seventeen to twenty bioelements: C, H, N, O; S and P; Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-; the trace elements, Mn, Fe, Zn, Cu, Co (I, Mo, B, Al, V)
   3. About 36 key organic molecules: glucose, ribose, deoxyribose, neutral fat, phospholipid, 20 amino acids, 5 nucleotides

VIII. Macromolecules
   1. Proteins
   2. Nucleic acids
   3. Nucleoproteins; viruses; bacteriophage

IX. Energetics of chemical reaction
   1. Thermodynamics: free energy, heat of reaction, entropy
   2. Temperature, molecular activation, and reaction rate

X. Enzymes and catalysis

XI. Cellular energetics
   1. Fermentation
   2. Respiration
   3. Hexosemonophosphate (HMP) cycle
   4. Photosynthesis
   5. Chemoautotrophy: the nitrogen cycle

XII. Organization of the cell, microscopic and ultramicroscopic

XIII. Mitosis and meiosis

XIV. Classical genetics
   1. Mendel's laws; linkage and crossing-over; chromosome mapping
   2. Sex determination
   3. Heteroploidy and polyploidy; chromosomal balance

XV. Fine structure genetics
   1. Recombination in bacteriophage
   2. Protein and nucleic acid synthesis and coding
   3. The molecular basis of mutation

XVI. Embryonic development
   1. Fertilization and cleavage
   2. The early embryo: vertebrate, invertebrate, higher plants to seed formation
   3. Differentiation
      a. induction
      b. nuclear changes
      c. nuclear-cytoplasmic relations

XVII. Endocrine control and hormones
1. General nature of hormonal action
2. Hormonal control of the sexual cycle in animals
3. Hormonal control of plant growth and development

XVIII. Physiological mechanisms: structure and function
1. The nervous system
   a. Nerve: structure, membrane potentials, the nerve impulse, spontaneous activity
   b. Receptors: generator potentials
   c. Nervous integration: synapses, reflex arcs, organization of the spinal cord, autonomic nervous system, brain
2. Muscle
   a. Muscle structure and function
   b. The chemistry of muscular activity
3. Digestion
   a. The course of digestion; enzymes
   b. Absorption, transport of food
   c. Role of the liver
4. Osmotic and ionic balance
   a. Kidney structure and function
   b. Ionic composition of blood and tissues
   c. Regulation of pH
5. The blood
   a. Blood cells and plasma
   b. Immunological reactions
   c. Individuality: problems of transfusion and organ transplantation
   d. Respiratory pigments: transport of oxygen and carbon dioxide

XIX. Evolution and its mechanisms
1. Time scale of evolution; major events in animal and plant evolution
2. Mechanisms of evolution: natural selection, artificial selection, sexual selection
3. Biochemical evolution
   (For traditional discussion of mechanisms of evolution and phylogeny we rely principally upon the reading.)
4. The evolution of man

It may be helpful also to say something of our laboratory facilities. Our introductory laboratories formerly had been furnished only with very low tables, bearing only microscope lamps, supposed to facilitate long hours of microscopy, and incidentally to keep the students fixed in position. This kind of thing has been more or less standard laboratory furniture for biology courses in the past.

In the present course we have stand-up benches, with adjustable stools for when the student must sit. The benches are in double rows, back to back, with facilities and a drain running down the middle. The facilities at each place include water outlets (one equipped with an aspirator), electricity, and gas. At the end of each double pair of benches is a large sink, for washing up and other uses.

The stand-up benches are important. They do not prevent microscopy, which seems to go as well on high stools and benches as nearer the floor. On the other hand, our students are not fastened down. They move about a great deal during a laboratory session, talking with one another, seeing what other students are doing, frequently going to the blackboard to argue a point. This is of course just what we want. If one of our laboratory sessions seems inordinately quiet and orderly, we know that something is wrong and try to stir it up.

To assist instructors in setting up, we have appended lists of materials and apparatus at the end of each exercise. We reserve one afternoon per week, on which no laboratory sessions are held, for setting up and going over the week's work with the graduate assistants. It will probably not surprise the readers of this manual to learn that a number of the exercises involve procedures that were new to most of our graduate students, and indeed to most of the staff including the professor in charge. This is a symptom of what it means to be teaching the new biology.

The exercises likely to present special problems—notably those in microbiology and electrophysiology—have detailed appendices that include information on sources of materials, apparatus, and prices. The prices are as of 1961–62 and are of course subject to change.

When we first began to prepare this course, we asked advice of many persons, and examined many other laboratory manuals. We should like to thank all those who generously contributed their advice and information.

We should like also to express our deep appreciation to the National Science Foundation, which through a generous grant of funds gave us the opportunity to explore the possibilities in this type of instruction far beyond what would otherwise have been possible. We should like particularly to acknowledge our indebtedness to Dr. Bowen C. Dees, Assistant Director of the Division of Scientific Personnel and Education of the N.S.F., and to Dr. Charles A. Whitmer, Head of the Course Content Improvement Section. The help we have received from the National Science Foundation implies a public obligation which we gladly accept and are anxious to fulfill. We shall be glad to help in any way we can with the use of this manual and the institution of this type of instruction in biology.

It hardly needs saying, however, that we need more help than we can provide. The present contents of this manual represent little more than work in progress. We are anxious to improve it, and would be most grateful to hear from any of our readers their criticisms, suggestions for improving the present experiments, and suggestions of new experiments.

Cambridge, 1962

G. W.
P. A.
J. E. D.
J. H.
S. L.

WHY A BIOLOGY LABORATORY?

A Foreword to the Student

Science is an attempt to understand reality. The questions we ask, and the answers, are put into words, and we try to give the words the clearest meanings we can. But they are no substitute for reality. They always fall short of saying what needs to be said. Even after one has learned to talk easily about nature in certain ways, after the words and phrases and concepts have grown familiar, the contact with the thing itself is always surprising. It has a quality of newness and freshness; one feels that for the first time one really understands—or, what is at least as good, that one has never understood at all—that the familiar words had been concealing mysteries. Often it looks as though something were being explained, when in fact it is only being named. A lot of scientific terminology is of this kind. It does well enough in a world of words, but fails immediately in a world of things.

Nowhere is this as true as in biology. The word "life" itself balks all attempts to define it. The trouble is that whatever definitions of life we make are easily fulfilled with models that clearly are not alive. What we do about life is not define it, but *recognize* it. It would be an interesting experiment to see whether you could be fooled now; whether if we showed you a lot of different things, alive and dead, you would have trouble telling the one from the other.

In any case we hope you will do better after your experiences in this laboratory; better, not only in telling what is alive from what is dead, but in knowing what to expect of living things, what they do, how they behave, what they can tolerate, and what is likely to kill them. This is what biologists sometimes talk about as "the feel" of living organisms, something one gets only by living with them—by observing, playing with, and experimenting with them in their great variety, until one has developed intuitions of what kinds of things they do and don't do, and what one can do and not do with them. Scientists of all kinds—physicists, chemists, geologists, astronomers—are turning their attention to biology as never before; and this is a fine thing. Many biologists think, however, that what some of these visitors lack is just this "feel" for organisms. Sometimes they know the words, but make obvious mistakes or miss the point entirely, because they do not know living organisms and do not have useful intuitions about them.

Living organisms are made of molecules, and it is important not only to develop a "feel" for the organisms, but equally for the molecules that compose them. They are for the most part a special group of molecules, made almost exclusively of carbon, hydrogen, nitrogen, and oxygen—so-called organic molecules. All of them are interesting, and all have special properties; but particularly the big ones, the proteins and nucleic acids, have qualities of their own that set them apart to a degree from all other molecules. They are at once the largest and most complicated molecules we know. Here again the words fail. It is only by preparing and handling them, by learning what they will

tolerate by way of handling, and what destroys them, that we gradually acquire a "feel" for proteins and nucleic acids, just as one does for organisms. Indeed, the one greatly helps the other, for many of the basic properties of living organisms derive from their proteins and nucleic acids. Here again it is only long experience with these molecules in their great variety that develops the intuitions that give point and meaning to our concepts.

This is our aim in the laboratory, therefore—to make direct contacts with living organisms and with the molecules that compose them. A great Harvard biologist, Louis Agassiz, the founder of the Museum of Comparative Zoology, is often quoted as having said, "Study nature, not books." The statement is a little foolish if taken literally; for one thing, you have just read it in a book. I think he really meant that we should do both but wished to remind us that studying nature is a very different thing from studying books, and at times more reliable. In any case, our job in the laboratory is the study of nature itself.

We will pursue it there for its own sake, not merely to illustrate and amplify the content of the lectures. Indeed, laboratory work develops on its own, independently of the lectures; and you should approach it with this in mind. If something comes up in the laboratory that has not been mentioned at the lectures, as will happen regularly, master it then and there. We will try to help you in every way we can, but much of it is up to you. Know what you are doing in the laboratory at all times. No mistake would be as great as to go through a laboratory session in a state of confusion, hoping that some later lecture will clear it up. We hope that later lectures *will* make things clearer. In fact, we hope the whole course hangs together in that regard. But each laboratory experience must be met on its own terms, then and there.

One last word: your business in the laboratory is with living organisms and the molecules that compose them. This laboratory guide, your instructors, the instructors' questions, are all to help deepen and enrich that experience. They are not objectives in themselves. Come to the laboratory as a scientist, to put questions direct to nature. Experiment and observe generously, not just what we suggest, but whatever interests you. Try to raise your own questions; we will appreciate them more than the ones we ask you. This is your opportunity to have a meaningful experience with a lot of things you may never have in your hands again. Make the most of it.

A few technical matters

Notebooks. Get a three-ring loose-leaf notebook for the laboratory and a block of unlined paper on which you can take notes. Note down whatever is essential in your experiments, in good English and in good order, so as to give a clear and connected account of what you have done, your observations, and the results of your experiments. Whenever a drawing helps, make one. The point is for it to be clear and informative, not necessarily beautiful.

Don't copy out sections of this laboratory guide into your notes. Whatever you need to describe, put into your own words. Answer all questions.

The notes may be in pencil or in ink. Drawings, of course, are better done in pencil. Do not use a soft pencil for either notes or drawings, since it smudges. A No. 3 pencil is of about the right hardness.

Preparatory reading. At the beginning of each exercise you will find references to textbooks and often also to *Scientific American* articles. These should be read before you come to the laboratory. Often it would be useful for you to have a textbook in the laboratory with you, but only for reference, not for extensive reading. Read the directions beforehand on the experiment you are about to undertake, and try to get a good idea of what you will be doing and in what sequence. The better prepared you are on coming to the laboratory, the more you will get out of it.

The three books most commonly referred to in the preparatory reading are:

The Science of Biology, by Paul B. Weisz, McGraw-Hill Book Co., 1959 (referred to hereafter as "Weisz").

Bioliogy, by Claude A. Villee, W. B. Saunders Co., 1962 (referred to hereafter as "Villee").

Life, by G. G. Simpson, C. S. Pittendrigh, and L. H. Tiffany, Harcourt, Brace and Co., 1957 (referred to hereafter as "S.P.T.").

Numerous other books are referred to throughout the manual, where they are fully identified, as are others listed in the Bibliography at the back of the manual.

Scientific American articles are identified both by date of issue, and for the benefit of those who have access to the reprints issued by W. H. Freeman and Company, by reprint number.

Equipment. You will need dissecting tools: 1 scalpel, 1 pair of scissors, 1 pair of forceps, 1 dissecting needle, represent a minimum set. Students going on in biology may wish to purchase high-quality instruments and more of them; a large and a small pair of forceps, for example, and a large and small pair of scissors. You may also want a laboratory apron.

Cleaning up. Leave the laboratory as you find it, or better still, as you wish you had found it. Wash any dirty glassware and other equipment with detergent or other cleanser, using brushes when needed. Then rinse each article at least five times, so that no soap whatever is left. Carelessness in rinsing may spoil a later experiment.

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1 LIVING CELLS (I)

(Readings: Weisz, pp. 55–67. S.P.T., pp. 39–58 and 488–498. Villee, pp. 35–42. J. Brachet, "The Living Cell," *Sci. Am.* **205**, No. 3, pp. 50–62, Sept. 1961, Reprint No. 90.)

The cell is the minimum organization that displays the properties and processes that we refer to collectively as "life." We know life only in the form of living cells. They are called "cells" because each is enclosed in a continuous boundary, the cell membrane, and sometimes also a cell wall.

Some living organisms are composed of a single cell; others are multicellular. A multicellular organism may be composed of many different types of cell, each type playing a different role. A group of similar cells, specialized for a single type of function, is called a *tissue*. Between the unicellular and the multitissued organisms, we find a few multicellular forms that, because all the cells are of much the same type, we speak of as "colonial."

Most living organisms can be characterized clearly as plant or animal, though one tends now to recognize a third great kingdom, that of the protists, which includes all unicellular and colonial forms. Typical plant cells are characterized by a rigid cell wall, made largely of cellulose, and may contain organs of photosynthesis, the chloroplasts. Both plant and animal organisms may be uni- or multicellular, or colonial. Among the unicellular or colonial forms, in addition to those that are clearly plant (algae) and clearly animal (protozoa), there is an interesting group that does not fit easily into either category—the green flagellates.

We shall devote the first two laboratories to examining a variety of living cells and what they do. You will see that though they look very different from one another, they share many properties in common. Toward the end of the second laboratory, we will try to mimic some of their activities with simple inorganic models. The cells are made of molecules, much the same types of molecules in all living cells. Immediately after this work with living cells, we shall go to work with the molecules. It is a striking fact that the chemistry of living organisms varies much less than their anatomy.

CELLS OF MULTITISSUED ORGANISMS

Onion epidermis

Remove a fresh inner scale from an onion. With a scalpel and forceps strip off a layer of the epidermis from the *inner* side of the scale. Mount a piece in *tap water* on a slide, and with

forceps place a cover glass over it, putting one edge of the cover glass down first and then letting the other end down slowly so as to drive out all bubbles of air. Examine under the low power of the microscope. The epidermal cells of the onion are typical plant cells in that they consist of (1) a cellulose cell wall, (2) a thin layer of cytoplasm which lines the cell wall, (3) a nucleus, and (4) a large central vacuole. Observe as many features of the living cell as you can. Stain a piece of this tissue with aceto-carmine. This is a dye which stains basic proteins red, made up in 45% acetic acid which coagulates protoplasm (like cooking an egg). Sketch one cell, showing cell wall, cytoplasm, vacuole, and nucleus.

Human epidermis

Having seen some onion skin under a microscope, you may enjoy seeing your own. With a reasonably clean fingernail, or the blunt end of your scalpel, scrape the inside of your cheek lightly. Stir the scrapings into a drop of tap water on a slide, cover with a cover slip, and find the cells under low power. They will appear as small masses of colorless, granular material. Under high power, study their structure. This is a large, flat type of cell (*squamous epithelium*) that, as in the onion, forms tissue surfaces. Individual cells are best seen at the margins of a group. Note the cell membrane, junctures with neighboring cells, the granular cytoplasm, and the small, rounded, highly refractile nucleus, itself surrounded by a membrane. Sketch one cell. Compare the cell membrane with that of the onion cell; this is one of the features by which one distinguishes plant and animal cells.

Highly specialized cells: the *Elodea* leaf

Elodea is a flowering plant that grows in fresh water. Pluck a young leaf and mount it whole in tap water, top side up, under a cover slip. Under low power find a group of elongated cells near the midrib and toward the base of the leaf. The green structures are *chloroplasts*. They are the organs of photosynthesis, and the green chlorophyll and other pigments they contain absorb the light used in this process. In some cells you can observe a circulation of the protoplasm (cyclosis). If you find this, try the effect on it of changing the brightness of the light. Make a sketch of a single cell, showing the relation to its neighbors, and as much of the internal structure as you have seen.

You have already seen an example of the cellulose wall that typically encloses a plant cell, and of the delicate surface membrane that surrounds the cytoplasm of all living cells, the so-called plasma membrane. We shall take this opportunity to demonstrate the relationship between these structures in *Elodea* cells. When such a plant cell is laid in a salt solution more concentrated than its own cytoplasm, the salt solution draws water out of the cell, causing it to shrink away from the rigid cell wall, so exposing the plasma membrane.

Mount a whole *Elodea* leaf as above, and when you have a good field of cells in focus under the microscope, replace the tap water by a concentrated solution of sodium chloride (2 *M*). This is done by using a medicine dropper to place a single drop of the salt solution on the slide beside the cover slip, just making contact with its edge. Then touch the margin of liquid at the opposite side of the cover slip with a small piece of lens paper, so that the lens paper draws up the liquid, sucking the salt solution under the cover slip to replace the liquid you have withdrawn. When you have done this two or three times, the liquid under the cover slip will have been completely replaced by the salt solution. This is the general method used for changing solutions under a cover slip, so that their effects can be observed as the change progresses.

Potato

Cut a thin slice from a freshly cut surface of a potato tuber. Lay the section in a drop of water on a slide and examine under low power.

Stain by adding a drop of iodine-potassium iodide solution (I_2 + KI, 0.01 *M* each). Cover with a cover slip and look for the deep purple color that indicates the presence of starch.

COLONIAL ORGANISMS

Spirogyra, a green alga

Spirogyra is commonly referred to as pond scum. It tends to float on the surface in many fresh-water streams and ponds and is recognized by its bright green color and slippery feeling. Place a few filaments of *Spirogyra* on a slide in a drop of water. Cover with a cover slip. Under low power select a group of cells with regular, spiral, green chloroplasts. Examine under high power. Note the *pyrenoids* in the chloroplast. These are associated with starch formation. Where is the nucleus and how is it held in place? Sketch a cell showing the various structures you see.

Test for starch by adding to a strand of *Spirogyra* on a slide a drop of iodine-potassium iodide solution as above. Note on your sketch the structures that stain most deeply with iodine.

Volvox, a "colonial" green flagellate

A *Volvox* colony may contain several thousand cells, embedded at the surface of a gelatinous sphere, with the flagella—two per cell—directed outward. The cells are interconnected by delicate strands of protoplasm. (You will see the arrangements better if you stain by drawing a drop of methylene blue under the cover slip.) Moreover the cells vary in size, shape, and function. For both reasons this is more than a simple collection of cells; it represents a genuine approach to a differentiated, multicellular organism. Sketch a colony. Do not try at this time, however, to see very much of the structure of individual cells. You will be able to do that better with closely related unicellular green flagellates.

OTHER ALGAE

Your instructor may also have other algae in the laboratory for optional study, perhaps an example of such a stonewort as *Nitella;* perhaps a diatom, one of the golden algae; or a desmid; or such a unicellular green flagellate as *Chlamydomonas.*

* * * * *

What is the simplest cell you have seen today? What would you say of the simplicity of the organism of which it is a part? The complexity of a multitissued organism is achieved through the specialization, and concomitant simplification, of its individual cells. Does specialization always imply simplification?

A NOTE ON THE COMPOUND MICROSCOPE

Realms of dimension

In a development that stretched over nearly three centuries, the compound microscope brought biologists into a world of new dimensions. Their dissections had previously been concerned with the gross anatomy of tissues and organs. Now they could penetrate to cellular anatomy. This involved a leap in dimensions of about 1,000 times, roughly from the level of millimeters to that of microns (10^{-4} cm). A cell in a multicellular plant or animal is usually one to several microns in diameter, though some algae are enormously larger, and bacteria in general very much smaller. The limit of resolution in visible light, that is, the separation at which two points in the object are seen as two rather than as a single blob, is about 0.2 micron. No details finer than this can ordinarily be distinguished, no matter how fine the instrument.

Recently the electron microscope has permitted a further leap in dimensions of approximately another 1000 times, from microns to millimicrons [1 mμ = 10^{-7} cm = 10 angstrom units (A)]. This has brought us from microscopic to ultramicroscopic anatomy, from the

anatomy of cells to that of subcellular particles. It has also made the larger molecules visible, for 10 angstrom units corresponds to the diameter of a rather small protein. The larger proteins and the nucleic acids and viruses can readily be distinguished under the electron microscope, and at times even identified by their characteristic shapes.

To go further requires radiations of still shorter wavelengths (x-rays). Methods of x-ray diffraction, simple in principle though complicated and laborious in practice, permit us to determine the positions of the individual atoms in molecules. Here the limit of resolution is a fraction of an angstrom unit. With such methods the characteristic distances and angles between the atoms in molecules can be determined.

This is the last reach of anatomy. It is not only that biological interest does not penetrate further; that might change with time. It is rather that beyond this point, anatomy becomes indeterminable. Particles smaller than atoms are subject to the limitations of physical indeterminacy; it is impossible to assign definite meaning to their individual spatial relations.

So the domain of biological anatomy stretches over an enormous range, from gross anatomy to molecules, from centimeters to angstrom units, from the anatomy of the dissecting pan to that of x-ray crystallography. There are no sharp boundaries; and nowadays biologists must be concerned with the entire continuum.

Use of the microscope

A compound microscope is a delicate and expensive instrument. Treat it tenderly. Don't begin by twiddling knobs, and then trying to find out what you twiddled. Find out what to do *before* you do it. Your instructor, charts, booklets, this outline—all will help.

The following summary of practical directions will get you started:

(1) Pick the microscope up by its arm. Don't let it knock against anything, and set it down gently to avoid jarring its parts out of line.

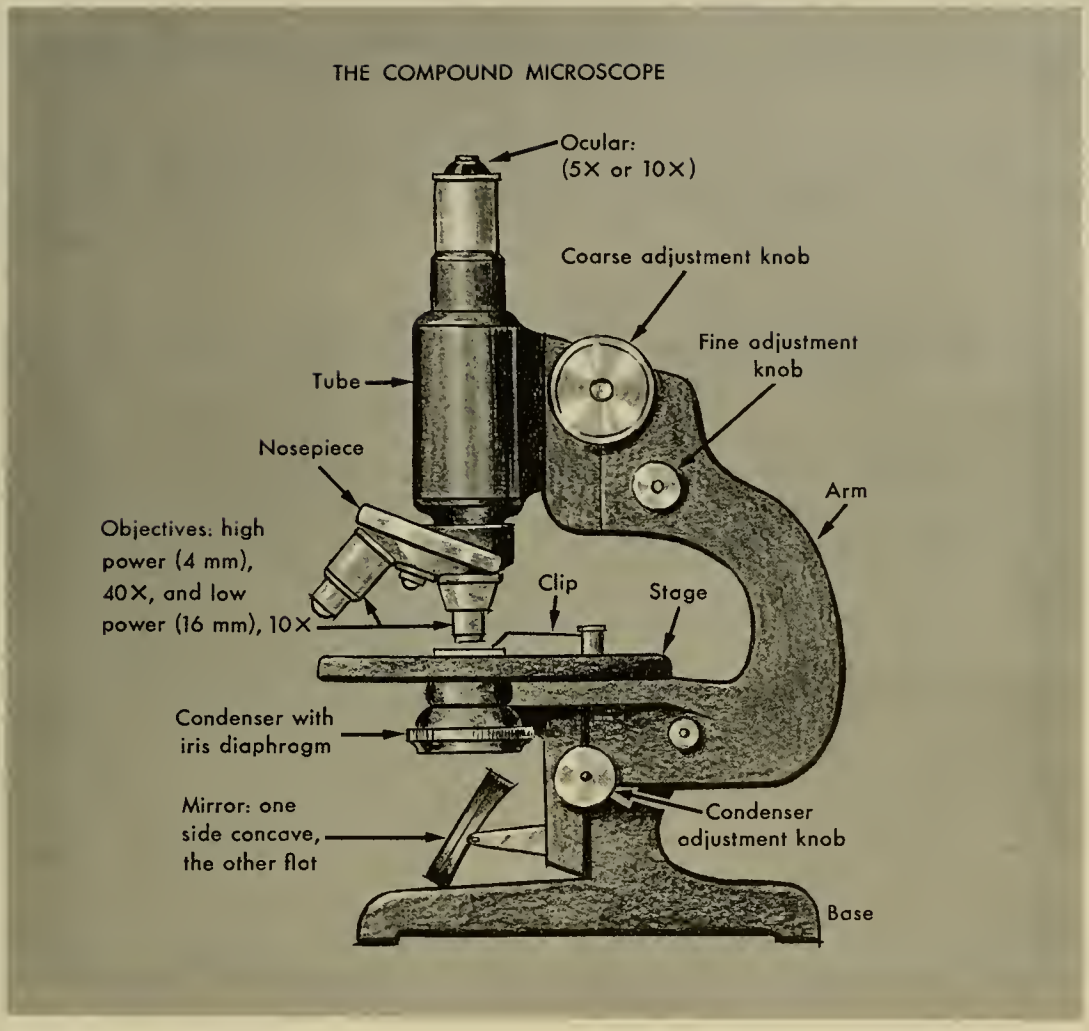
(2) Place the microscope on your desk with the arm toward you. Identify the ocular (eyepiece), nosepiece and objectives, the body tube, the coarse and fine adjustments for focusing, the stage, iris diaphragm, condenser (if present), and the mirror—one face of which is plane, the other concave—which reflects light through the opening in the stage into the objective.

(3) Revolve the nosepiece to bring the low-power (the shorter) objective into line with the body tube. A spring catch engages the nosepiece to hold each objective in its correct position.

(4) Adjust the mirror so that the concave side reflects light from your microscope lamp through the opening in the stage into the objective. If the light is too intense, close down the iris diaphragm until the brightness suits you.

(5) Always look at the object first under low power. Place a slide on the stage with its ends held by the spring clips (or in a mechanical stage), and position it so that the part you want to study lies at the center of the opening. Lower the tube until the bottom lens of the objective is about a quarter-inch above the slide. Now, while looking through the ocular, focus slowly *upward* with the coarse adjustment, until the image is visible. Continue to focus up and down with the fine adjustment until the image is sharpest.

(6) To use the high-power objective, first focus the object under low power and move the slide until the detail you wish to examine under higher magnification is almost exactly centered in the field. Turn the nosepiece slowly to swing the high-power objective into line. Watch meanwhile from the side, to see that this longer objective does not strike the slide or cover glass. Now focus slowly *upward* with the fine adjustment (the coarse adjustment is never used with the high-power objective). If no image is seen, carefully lower the objective until it almost touches the slide, then focus upward again. The point is to avoid any possible damage to the objective by permitting it to touch the slide. Finally, bring the image into sharpest focus by playing back and forth with the fine adjustment.



(7) When examining permanent slides, you may find it more comfortable to tilt the microscope toward you, at an angle with its base. For work with fresh preparations, always keep the stage of the microscope horizontal.

(8) It is good practice to keep both eyes open when looking through the microscope. You will soon learn to disregard the image from the "off" eye, and so will avoid the strain of holding that eye closed.

(9) Dirty lenses give poor results. Clean them only with the special lens paper which is provided, not with your handkerchief, Kleenex, or anything else. If after it is gently wiped with lens paper your microscope still does not yield a clear image, ask your instructor to help.

(10) The magnification of any combination of objectives and oculars is the product of the magnifications of the separate components. Thus a 10× ocular combined with the low-power, 10× objective yields a total magnification of 100 diameters. Each of the objectives is marked with its magnification and the distance from the object at which it yields an approximate focus. The low-power objective is in focus at about 16 millimeters above the object, the high-power (44×) objective at about 4 millimeters above the object.

(11) By moving your slide about gently while it is in focus, try to get used to the fact that the microscope reverses as well as magnifies every motion. In a little while you should have this under control. Also, in fresh preparations suspended in a liquid medium, do not be surprised to find all very small particles engaged in a continuous, random motion. This is called Brownian movement. It is caused by the fact that all objects suspended in a fluid medium are continuously bombarded by the molecules all about them; and sufficiently small particles are continuously knocked about by this bombardment.

EQUIPMENT

Throughout the manual these lists and the instructions which often accompany them are included for the use of the instructor.

Per student

compound microscope

Per 8 students

slides and cover slips
onion
potato
Elodea
cultures of *Spirogyra*
Volvox
other algae
iodine and potassium iodide in water (0.01 *M* each)
methylene blue solution
sodium chloride solution (2 *M*)

Per laboratory

charts on the compound microscope

MARINE BIOLOGICAL LABORATORY WOODS HOLE LIBRARY MASS

II LIVING CELLS (2); CELL MODELS

UNICELLULAR ORGANISMS

Paramecium, a ciliate

This is one of the commonest fresh-water protozoa. It is found in many pools, where it feeds on bacteria which, in turn, feed on decaying vegetation. The ciliates are the most complexly organized protists, at the opposite extreme from the Rhizopods. Indeed, because they present such a remarkable differentiation of structures and activities, it is hard to remember that these organisms are single-celled. In deference to their unicellularity, we speak of their organs as "organelles."

Put a small drop of *Paramecium* culture on a slide, and add about an equal drop of 4% methyl cellulose. Making the medium viscous will slow down the *Paramecia* so that you can observe them more easily. Carefully cover with a cover slip, supported on small broken pieces of another cover slip, and study under low and high power. Note the minute, whiplike cilia, whose regular, synchronized beat propels the animal through the water. Note the differences in length of cilia in different regions of the body; where are they longest?

Observe the two clear pulsating structures, the contractile vacuoles, near each end of the body. What functions do they serve? What human organ performs analogous functions?

Note that the *Paramecium* is asymmetrical. Note its "mouth," a groove or depression leading to a funnel-shaped gullet, at the end of which food vacuoles form.

Unicellular organisms ordinarily reproduce by fission, whereby a mature cell divides to form two equivalent daughter cells. Each individual could in this way become the origin of an immortal line, perpetually renewing itself by repeated division. If reproduction were perfect, that would do well enough, but as in any complicated form of life, aging processes occur. The genetic material of all cells is subject to random changes, called mutations. Aging is in part the result of the accumulation of mutations, which are usually deleterious.

For this reason there is great advantage in some arrangement that permits the individuals of any stock of organisms to mix their genetic material from time to time, so that out of all possible combinations, individuals emerge that possess particularly advantageous constellations of genetic characters. Sexual reproduction is such a device for regularly mixing genetic material. Such ciliates as *Paramecium*, though usually reproducing by fission, at times interpolate another process, a form of sexual reproduction, called *conjugation*. Two mature *Paramecia* join together side-to-side, exchange genetic material, separate, and then resume asexual reproduction by fission (see S.P.T., pages 491–492).

Identical *Paramecia* ordinarily do not conjugate. Conjugants, even though they may have

developed in the same culture, are heritably different from each other. We speak of such different strains as different *mating types*. At least 28 mating types are now known. These occur as 14 complementary pairs. Fertile conjugations occur only between individuals of complementary mating types.

We shall examine conjugation in two complementary mating types of *Paramecium aurelia*, Types XIII and XIV. Place 3 drops of Type XIII culture in the left depression and 3 in the center depression of a 3-depression glass slide. Place 3 drops of Type XIV culture in the right and center depressions. The right and left depressions will serve as controls for what happens in the center depression, where both types have been mixed.

When two mating types are compatible, as are these, the individuals first clump, their cilia sticking together. At this stage a narrow space can still be seen between them. After a short time, the *Paramecia* pair off, the mating individuals uniting side by side, and the pellicles fusing. It is at this stage that exchange of genetic material (haploid gametic micronuclei) takes place. Conjugation goes on for several hours. Then the mating individuals separate, and each resumes reproduction by fission.

After you have observed the original clumping, set the slide aside and go on with the rest of the exercise. After about an hour, a few pairs should have separated. Find such pairs and make a quick sketch of what you can see.

Euglena, a green flagellate

Place a drop of *Euglena* culture on a clean slide. Add a drop of 4% methyl cellulose. Cover with a cover slip, and observe, first under low, then under high power. Note the whiplike flagellum (often better seen by dimming the light), the chloroplasts, and the eye-spot, which seems to be a genuine light-receptor, guiding the motion of the organism toward or away from the light. Sketch one cell and its parts.

The green flagellates are hard to classify. Zoologists include them among the one-celled animals, the protozoa; botanists among the algae; or both avoid the issue by calling them "protists." What are their plant, and what their animal characteristics? One characteristic of typical plants is that they can incorporate inorganic nitrogen (nitrates, ammonium salts), whereas animals require their nitrogen in organic form (e.g., amino acids). On the basis of this criterion, how would you set up an experiment to classify *Euglena?*

Ameba, a Rhizopod (Sarcodine)

This is the famous protozoan whose name has become a household word for the simple and listless among living organisms. Put a drop of ameba culture on a clean slide, and observe with the naked eye against a dark background. The amebas can be seen as whitish dots about as large as a pinpoint. Look at them under low power, *without a cover slip*. See how they move by means of outward bulgings of the cytoplasm (pseudopodia = "false feet"). Watch carefully the formation of a pseudopod, and the associated flow of cytoplasm. Distinguish in the cytoplasm a clear outer layer, an inner, granular mass, and the single, ovoid nucleus. Make a series of 6 outline sketches of a progressing ameba, recording the time of each sketch.

For study under high power, it is preferable to use a cover slip. So that this will not crush the ameba, place four bits of broken cover slip around the drop of culture, and set the cover slip on these. Under high power observe the granules, food vacuoles, nucleus, contractile vacuoles, and pseudopodia.

It must be plain to you now that the ameba has been maligned. It is not as simple as it looks. It packs more into a small space than anything yet designed by man. It can move, react to stimuli, reproduce, adapt to the environment (how?), ingest solid food, excrete waste, and regulate its water content. How do you manage to do more? With all that churning up of its contents, how does the ameba keep its functions sorted out and balanced?

Most amebas are free-living, but one notorious human parasite in this group is *Endameba histolytica*, which causes amebic dysentery. It is easy to think of such Rhizopods as the ameba as primitive and as ancestral to other types of protozoa; but an interesting argument considers the flagellates to be the most primitive protists, and derives the ameba from them (cf. Weisz, pp. 664–667).

Reactions of *Paramecium* to its environment

To another drop of *Paramecium* culture on a fresh slide, add a *small* drop of a suspension of powdered carmine before adding the methyl cellulose. Observe how the granules of carmine accumulate in a food vacuole at the end of the gullet, which is pinched off and then pursues a definite course around the cell. Follow one such vacuole through its circuit. Unlike the ameba, *Paramecium* has a special area which serves for the egestion of solid wastes. This anal spot is on the surface, about level with the posterior end of the gullet. It can be detected only during the act of egestion. You may be able to see the elimination of carmine particles through the anal spot. (*Note:* If the methyl cellulose does not slow the organism sufficiently for these observations, use in addition or instead a bit of lens paper laid in your preparation.)

Paramecia propel barbed, harpoonlike *trichocysts* when disturbed. Place a large drop of *Paramecium* culture on a slide, and put a very small drop of ink next to it but not touching it. Now bring the two drops into contact, put a cover slip over them, and quickly examine under low power. Note what happens when a *Paramecium* swims into a blue zone.

In a fresh preparation made up with lens paper and not containing methyl cellulose, note how *Paramecium* reacts to obstacles. Do you see what is meant by its "trial-and-error" behavior? Can a *Paramecium* back up? How do you suppose it does so? How does it synchronize and integrate the beating of its cilia? Has it a nervous system? Of what could a subcellular nervous system be composed?

Other protozoa and algae

Your instructor will also have available some pond water containing protozoa and algae other than those already studied. We are not interested in identifying them except in the roughest way; but they are interesting to find and watch, and to assign to the major groups. How many different kinds do you see? Are organisms present that are *not* unicellular? Of what types? (Your instructor and reference books will help you answer these questions.)

* * * * *

You have now seen a wide range of living cells, from the comparatively simple ones to the exceedingly complex. With simplicity and complexity we often associate such terms as "primitive" and "advanced," or "lower" and "higher." Would you say that *Paramecium* is a "lower" organism? Does it seem "primitive" to you? Does it seem more "primitive" perhaps than an onion or a man, of which you have seen epidermal cells? Which is "higher" or more "advanced," *Euglena* or the ameba which may be derived from similar flagellates?

MODELS OF LIFE

A discouraging thing about defining life is that once one has made a definition, it is easy to construct a model that satisfies the definition, yet clearly is not alive. Such models are themselves instructive, because they sometimes present much simpler systems that display properties exhibited by living organisms in ways that permit closer analysis, and suggest physical and chemical bases for these phenomena in the living organisms themselves. Such an application of models can be misleading; one needs to judge carefully how far to pursue a model, and when to leave it.

In any case, the model we are about to examine should be thought of in two ways: as a demonstration that some phenomena of living organisms are easily reproduced in inorganic

systems; and to raise the question whether the model and the organism behave similarly for the same reasons.

An artificial "ameba"

Into a clean Syracuse watch glass laid on a piece of white paper pour dilute nitric acid to a depth of about $\frac{1}{8}$ inch. Into this introduce a drop of mercury about $\frac{1}{4}$ inch in diameter; the mercury is best introduced by putting the tip of the pipet that contains it under the surface of the nitric acid. *Be careful not to spill any mercury.* Drop a crystal of potassium dichromate about $\frac{1}{8}$ inch in diameter or somewhat larger into the nitric acid about $\frac{3}{4}$ inch from the mercury drop. You will immediately see the potassium dichromate beginning to dissolve in the nitric acid and diffusing from the crystal in all directions. As the boundary of this yellow diffusion zone reaches the mercury drop, things begin to happen. Watch this for a time and describe the phenomena you see. Are the motions you observe comparable in any way with the mode of locomotion of an ameba? Have you observed anything resembling cell division?

The physico-chemical basis of this behavior is as follows. Mercury has an exceedingly high surface tension, the highest of any known liquid, and for this reason assumes an approximately spherical form on a surface, though flattened by its own weight. The potassium dichromate in nitric acid oxidizes the surface of the mercury, lessening momentarily the surface tension at this point, causing a local outflow of mercury. Such points of oxidation, distributed asymmetrically over the surface from moment to moment, lead to the motions and cleavages you have observed.

(Go on watching this experiment as long as you like. When you decide to clean up, be sure not to drop any mercury on the floor or to let any run into the sink. There will be a container available into which to pour it. Mercury blocks and rots plumbing; but much more serious is mercury spilled around the room or on the floors. There it enters the dust and may be inhaled or otherwise absorbed by the body in this form. Since it is not readily excreted, the body tends to accumulate it, and in larger amounts it can produce very serious disturbances. Make certain, therefore, that mercury is not spilled, and that if any is spilled by accident, it is immediately picked up. Any of it that is on the floor can be brushed into a dust pan by using a wet brush.)

Ingestion, digestion, excretion

In feeding, a protozoan exhibits some degree of choice. Ordinarily a protozoan takes in some objects and not others. Having taken in a particle of potential food, the cell digests it in part and excretes what remains. The process of digestion is well understood, but the mechanisms by which the organism ingests some objects and excretes others are only partly understood. In performing the following experiment we should like you to note what analogies to these processes it presents, and to ask yourselves to what degree the simple mechanisms it involves are related to the comparable phenomena in living cells.

Put about 2 inches of distilled water into a 6-inch test tube, and drop into this 6 or 7 drops of chloroform. Swirl the water in the test tube and wait a minute for the chloroform to coalesce at the bottom to form a single more-or-less spherical drop about $\frac{1}{8}$ inch across. Draw a clean piece of glass rod, about $\frac{1}{8}$ inch thick, to a fine tip. Now attempt to insert the tip of the rod into the drop of chloroform. Does the drop accept it? Now wipe the rod dry, and dip the very end into a solution of shellac. Blow on it until it is dry. Now try again to make it enter the drop of chloroform, watching closely what happens. Does the drop accept it in the first moment? later?

Such a drop of chloroform under water, like that of mercury, approximates a spherical shape. It does so not so much because of its own surface tension but because of the surface tension of the water that surrounds it. Surface tension is a force well described by its name: the molecules of a fluid attract one another more or less

strongly depending upon the substance of which it is composed, and at the surface, where this attraction is all directed inwardly, it produces a tension which tends constantly to contract the surface to a minimum. This is why such fluids tend when possible to assume the spherical form which presents the smallest possible surface for a given volume. Any distortion from the spherical form is resisted by the surface tension.

Glass is, of course, insoluble in chloroform, and the introduction of a glass rod would increase the surface of the drop. Its resistance to this increase of surface is the force that tends to expel the rod from the drop, or vice versa. The coating of the rod with a substance soluble in chloroform (e.g., shellac) entirely changes these relationships, since a coated rod no longer offers an incompatible surface to the chloroform, but instead a substance ready to enter the same phase with it. As a result, the drop now accepts the glass rod. In the happy event that the size of the chloroform drop and the amount of shellac on the rod come out about right, one might observe that after accepting the shellac-coated rod for a while, the drop spontaneously moves apart from it again. The explanation is that the drop has finished dissolving the shellac off the rod and now rejects the rod itself, as originally. One can think of this as a model of a cell taking in an object which is partly food and partly indigestible, digesting off the food, and excreting the remainder. One sees also in the behavior of this drop that through simple forces of surface tension, the surface of separation between two immiscible phases (water and chloroform in this case, but equally water and air, or any others) forms a kind of skin with special properties, an approach to a surface membrane. This resists penetration by substances which it cannot dissolve or with which it cannot react, and on the other hand it is readily penetrated by substances that it can dissolve or with which it can react.

EQUIPMENT

Per student

compound microscope
slides and cover slips
Syracuse watch glass
piece of glass rod, 6 to 8″ long, about $\frac{1}{8}$″ thick
6″ test tube
medicine dropper

Per 4 students

dropping bottle of 4% methyl cellulose
package of lens paper

Per 8 students

dropping bottle cultures of *Paramecium* (including *Paramecium aurelia* Types XIII and XIV), *Euglena*, ameba, and pond water
dropping bottle of blue ink
dropping bottle of carmine suspension
dilute nitric acid (about 1 *M*; dilute about 60 ml of concentrated acid to 1 liter)
clean mercury (10 cc)
potassium dichromatic crystals (10 gm)
chloroform (25 cc)
white shellac (10 cc)

Per laboratory

reference books containing pictures of various microorganisms
pure-line cultures of opposite mating types suitable for demonstrating conjugation (*Paramecium bursaria*) can be obtained from the General Biological Supply House, 8200 So. Hoyne Ave., Chicago 20, Ill.

III CHEMICAL COMPONENTS OF CELLS: MACROMOLECULES OF YEAST AND THEIR SUBUNITS (I)*

(Readings: Weisz, pp. 17–38 and 149–156; Villee, pp. 26–31. F. H. C. Crick, "The Structure of the Hereditary Material," *Sci. Am.* **191**, No. 4, Oct. 1954, Reprint No. 5. P. Doty, "Proteins," *Sci. Am.* **197**, No. 3, Sept. 1957, Reprint No. 7.)

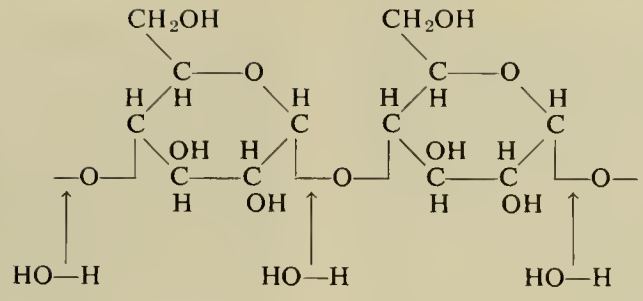
A cell lives by virtue of its composition and organization. Both are unique: the composition in large part because of the universal presence of certain classes of very large molecules, so-called macromolecules, the largest and most complex in all chemistry; and these are responsible also for many of the most distinctive features of cellular organization and behavior.

Our approach to the macromolecules is enormously simplified by three circumstances: (a) All of them fall into three great classes—polysaccharides, nucleic acids, and proteins—common to all cells, and sharing common properties within each class. (b) Each type of macromolecule is composed of a limited number of repeating subunits, bound together to form long chains. The subunits of the polysaccharides are sugars; those of nucleic acids, nucleotides; and those of proteins, amino acids. Rather than dealing with the individual atoms of which these molecules are composed, which may run into many hundred thousands, we deal with the much smaller numbers of subunits. (c) In all types of macromolecule, the subunits are bound to one another through the same device, the elimination of a molecule of water between each pair. Conversely every macromolecule may be broken down into its subunits by the reverse process, the insertion of a molecule of water between each pair. The latter process is called *hydrolysis*. *Digestion* is a series of such hydrolyses, catalyzed by enzymes in the digestive system, which cleave all the macromolecules of the food into their constituent subunits:

Sugars	Synthesis $-(n-1)\ H_2O$ →	Polysaccharides (glycogen, starch)
Nucleotides	← $+(n-1)\ H_2O$ Hydrolysis, Digestion	Nucleic acids
Amino acids		Proteins

In this and the next laboratory session we will separate the major types of macromolecules

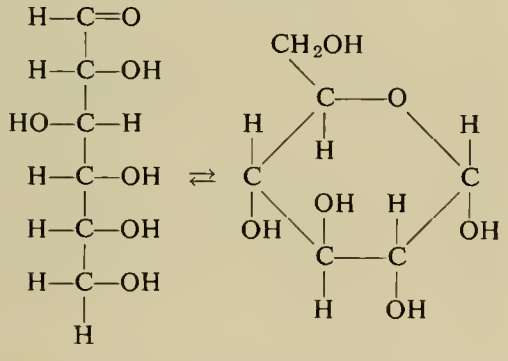
*An alternative or supplementary pair of exercises on the biochemistry of milk will be found in Appendix C.



from a cell, and learn something of their properties and the units of which they are composed. For convenience we shall work with yeast cells, but the results would be much the same if we used any others.

The simplest of the macromolecules are the polysaccharides. They include the starches and glycogens, forms in which the cell stores sugar for future use; and such inert structural polysaccharides as cellulose, the principal component of plant cell walls. Each of these molecules, though very large, is made of a single repeating unit, glucose.

Glucose contains 6 carbon, 12 hydrogen, and 6 oxygen atoms, so that its *empirical formula* is $C_6H_{12}O_6$. What is much more important is the arrangement of these atoms in the molecule, the so-called *structural formula:*



These are two forms in which glucose exists at all times, in equilibrium with each other. The straight-chain form at the left is present in minor amounts, but exposes the aldehyde (HC=O) reducing group upon which the test you will perform next week depends. The ring structure at the right is by far the more prevalent.

A starch or glycogen is formed by stringing hundreds or thousands of glucose molecules together by eliminating a molecule of water between each pair, as shown above. That is, $n(C_6H_{12}O_6) \rightarrow [(C_6H_{10}O_5)_n \cdot H_2O] + (n-1)H_2O$, in which n is the number of glucose units involved. [Why is the number of water molecules eliminated $(n - 1)$?]

Such a chain, several hundred glucose units in length but unbranched, is the component of starch that yields a blue color when treated with iodine. A second component of starch is formed of similar chains, but highly branched; and glycogen, the characteristic storage polysaccharide of animal tissues and yeast, consists entirely of such highly branched chains. The highly branched polysaccharides yield brownish or reddish colors when treated with iodine.

The nucleic acids are composed of units called nucleotides, tied together to form long unbranched chains, thousands of nucleotide units long. Each nucleic acid contains four different nucleotides; and since so many of these units are involved, and they can be arranged in any sequence along the chain, it is possible to construct in this way an enormous number of different nucleic acids. Such variety is needed, for among other things nucleic acids form the functional components of the genes, and it takes a lot of genes to account for the heredity of all living things.

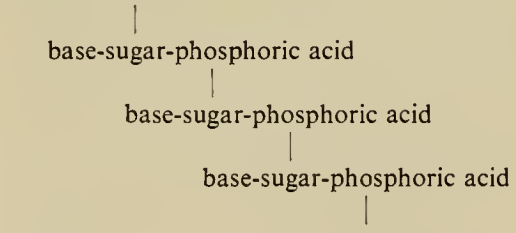
Any cell, even one that looks as simple as yeast, contains a large number of different nucleic acids, representatives of the two great families, ribonucleic (RNA) and deoxyribonucleic acid (DNA). Each, as already said, is made of four different nucleotides; and each

nucleotide is itself composite, being made of a nitrogenous purine or pyrimidine base, a 5-carbon sugar (ribose in RNA and deoxyribose in DNA), and phosphoric acid, united to one another by the same principle of elimination of water between them. The hydrolysis of nucleic acid not only may cleave the nucleotides, but may sever all these linkages, leaving us with a mixture of the free nitrogenous bases, 5-carbon sugars, and phosphoric acid. The acid hydrolysis that we will perform releases nearly all the purine bases, but only a small fraction of the pyrimidines.

The ultimate components of the two families of nucleic acids are:

RNA		DNA
adenine, guanine	purines	adenine, guanine
cytosine, uracil	pyrimidines	cytosine, thymine
ribose		deoxyribose
phosphoric acid		phosphoric acid

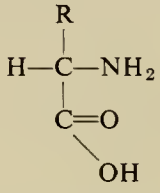
The first four substances named in each column are the nitrogenous bases. A nucleotide can be written: base-ribose-phosphoric acid, the nucleotides of each nucleic acid differing only in their bases. The fundamental arrangement of nucleotides in nucleic acid is:



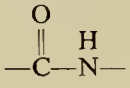
where each bond represents a point at which molecules have been united by elimination of water, and conversely can be hydrolyzed by the insertion of water.

The third class of macromolecules, the proteins, is composed of up to 20 different amino acids, joined together to form chains hundreds to thousands of amino acids in length. Since proteins are of many sizes, and their amino acids can be united in any proportions and in any sequences, almost an infinite variety of different proteins can exist. Living organisms take full advantage of this possibility, for as far as we know every living species, animal and plant, contains specific proteins different from those of all other living species. Proteins account for much of the internal structure of cells, and all known enzymes are proteins.

An amino acid has the general formula:

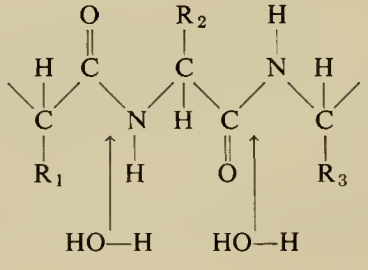


in which $—NH_2$ is the amino, —COOH the carboxyl (acid) group, and R may be any one of 20 different groups (—H, $—CH_3$, $—CH_2OH$, etc.). Amino acids are joined to one another by taking out a water molecule between the $—NH_2$ group of one and the —COOH group of its neighbor. The joint that results,



is called a *peptide bond.* Groups of amino acids linked together in this way are called polypeptides until they get big enough to be called proteins.

The general arrangement of amino acids in a segment of polypeptide or protein follows. The insertion of molecules of water at the places indicated by arrows hydrolyzes the structure into its constituent amino acids:



A WORD ON MOLECULAR STRUCTURE

Molecular structure is anatomy carried to the level of small dimensions. We hope that by now you would have no difficulty recognizing an ameba or a *Paramecium* when seeing one under the microscope. In exactly the same sense you should learn to know a sugar, fat, or a section of a protein or nucleic acid molecule from its molecular appearance. Molecules are three-dimensional structures, with characteristic anatomies upon which many of their properties depend. Some violence is done by the habit of portraying them on the plane surfaces of paper and blackboards; yet even such two-dimensional representations are useful and recognizable. After all, this is no greater violence than is involved in pictures of animals and plants.

Fortunately, however, we can do something much better, and we hope you will take full advantage of it. You will find in the laboratory sets of molecular models, from which you can construct sugars, fats, representative sections of proteins and nucleic acids, and many other types of molecule that we encounter in this course. With these models you can also inquire into such interesting and important matters as optical activity, associated with the right- or left-handedness characteristic of many of the organic molecules found in cells.

It would be altogether wrong to deal with these molecules simply as words, the names of abstractions. Use this opportunity in the laboratory to handle them and look at them as things, which is what they are. Make yourselves models of glucose, and join them together by taking out molecules of water between them, as in polysaccharide formation; then split them apart again by inserting water molecules, as in hydrolysis. Similarly construct a polypeptide chain from a few generalized amino acids, and see what it means to hydrolyze such a chain, the process catalyzed by such protein-hydrolyzing enzymes as are found in pancreatic extracts.

From now on whenever you have a little free time in the laboratory, one good thing to do with it is to construct molecular models, and carry out reactions with them. This is fun to do, it will help you greatly, and it is as close to synthetic organic chemistry as many of you will ever come.

One last word about these models. They are probably of a relatively inexpensive type, that represents fairly correctly interatomic distances and bond angles. The little balls that represent the atoms, however, show only the relative positions of the centers of those atoms, not the space they occupy. In a more correct and much more expensive type of molecular model, which tries in addition to represent the space-filling properties of the atoms, one sees that molecules are much more solid structures. In such a more correct model, for example, the six-membered ring of glucose is seen to have almost no hole in the middle.

EXPERIMENTAL PROCEDURE

Yeasts are a unicellular type of fungus which reproduces by budding. The species of yeast we shall use, *Saccharomyces cereviseae*, serves many human uses. Different strains of it have been developed as baker's yeast, for raising dough; brewer's yeast, for fermenting malt to make beer; and various types of wine yeast. We shall be working with baker's yeast, which ordinarily comes in cakes with starch as a binding material. We have carefully washed the starch away, leaving a clean suspension of yeast cells with which to work.

Stir a pinch of yeast into 1 ml of glucose medium, and set it aside. Toward the end of the laboratory period, when you have time, make a slide of a drop of this, and examine the budding cells under the high power of the microscope. During this interval the yeast will have begun to ferment the glucose, and you will see the bubbles of carbon dioxide which is one of the products.

Our work in the laboratory will involve a number of processes that are new to many of you: centrifuging, neutralization of acids with

bases, and dialysis. After describing the procedure, we shall discuss each of these processes. That discussion is an integral part of the procedure, so be sure to read it before you begin work. We will begin with a general account of the procedure, and then give explicit directions in the form of a flow sheet.

Yeast cells are enclosed in a tough, cellulose-like outer wall. The wall is made of a polysaccharide called glucan, which contains only glucose units, but bound to one another differently than in cellulose, starch, or glycogen. The first operation is to break the cell walls by grinding the yeast with sand in a mortar, releasing the contents of the cells.

Extraction and hydrolysis of glycogen

The cell contents are stirred into trichloracetic acid solution (TCA) which dissolves the glycogen, leaving the nucleic acids and proteins as solid particles in suspension. This suspension is decanted from the sand, and the solid material separated off by centrifuging.

The glycogen is precipitated from the solution with ethyl alcohol, and this precipitate separated off by centrifuging. It is redissolved in 1 *N* hydrochloric acid (HCl), and this solution is divided in halves. One half is immediately neutralized with 1 *N* sodium hydroxide (NaOH), to prevent hydrolysis. The other half is heated for 30 to 60 minutes in a bath of boiling water (i.e., at approximately 100°C), and then is neutralized in the same way. The heating in acid solution hydrolyzes the glycogen completely. Each half is now placed in a dialysis sac, and the sacs are suspended in test tubes containing distilled water, and stored in a refrigerator until next week, when we will test for the presence of glycogen and glucose inside and outside the sacs.

Extraction and hydrolysis of nucleic acids

The solid residue of the yeast cell contents after the removal of glycogen contains nucleic acids and proteins. The nucleic acids are extracted into strong sodium chloride solution (NaCl), by stirring and heating in a boiling water bath, leaving an insoluble residue of coagulated proteins. The proteins are separated off by centrifuging, and the nucleic acids in the supernatant are precipitated with ethyl alcohol. This precipitate is collected by centrifuging, and dissolved in 1 *N* sulfuric acid (H_2SO_4). The solution is divided into halves, and one half heated for 30 to 60 minutes at 100°C to hydrolyze. Then both solutions are neutralized with barium hydroxide ($Ba(OH)_2$). The salt that results from the neutralization, barium sulphate ($BaSO_4$), precipitates out. The reason for acidifying with H_2SO_4 in this case, and neutralizing with $Ba(OH)_2$, is to get rid of this salt on neutralization because the paper chromatography of these solutions that you will carry out next week goes much better in the absence of salt.

Hydrolysis of protein

We are now ready to deal with the coagulated proteins. It would take many hours of boiling in strong acid or alkali to hydrolyze them. Instead, we perform this hydrolysis rapidly and at room temperature by using enzymes, as do living systems. We shall use a mixture of protein-digesting enzymes from a mammalian pancreas, which in life would have delivered this mixture of enzymes to the small intestine.

Small portions of the solid protein residue are transferred into each of two test tubes. One is stirred into a buffered solution of pancreatic enzymes, the other into a solution containing the buffer alone, to serve as control. Both will be stored by your instructor until next week, when their contents will be analyzed by paper chromatography.

DOING AN EXPERIMENT

The way to go at a job such as this, whether it is simple or complicated, is to read through the instructions and then make a plan of attack, in which you try to see yourself going through

the whole business. That is a very important part of getting ready to do an experiment. If you can see in your mind's eye just what you'll be doing and how you'll be doing it halfway through—whether, for example, you'll be holding the test tube in your right or your left hand at that moment—then you are ready to go to work. So your first job after reading through the above procedure and the further discussion of the manipulations below is to make yourself a schedule of just what you expect to do and when. It might come out somewhat as follows:

(1) Grind cells, extract with TCA, centrifuge.

(2) Precipitate glycogen, extract and precipitate nucleic acid.

(3) Centrifuge both preparations.

(4) Get both nucleic acid and glycogen samples ready to hydrolyze. Put into boiling water bath together.

(5) Prepare the protein hydrolysis.

(6) Look at the yeast.

(7) Neutralize the acid hydrolysates and start the dialysis of glycogen.

THE MANIPULATIONS

Centrifuging

In its most primitive form a centrifuge might be a boy whirling a bucket of water in circles around his head, which as you know can be done without spilling any water. If the water had small particles of sand suspended in it, this motion would make them settle faster to the bottom of the bucket. In its most complex form, an ultracentrifuge spins quartz tubes in an evacuated chamber at tens of thousands of revolutions per minute, developing forces well over 100,000 times gravity. Under these circumstances macromolecules, being somewhat denser than water, are sedimented. Your centrifuge operates in between these two extremes. Its maximum rate is about 3000 revolutions per minute (rpm), and you will always use it at its top speed.

A centrifuge is a potentially dangerous instrument, and certain precautions must be observed even with such relatively slow types as you are using:

(1) Use only plastic tubes, and do not fill higher than about $\frac{1}{2}$ inch from the top.

(2) Each tube in the centrifuge must be balanced against another of the same weight just across from it. In your experiment it will be enough to have both these tubes contain the same volume of solution, gauged by eye. In faster centrifuges it is necessary to balance the pairs of tubes, preferably with their cups, against each other on a sensitive balance.

(3) Place the centrifuge well away from the edge of the work table, and be sure that it is level. Otherwise it may creep off the table while running.

(4) Close the lid before starting the centrifuge and leave it closed until the centrifuge has stopped spinning. Let it stop by itself; do not brake it by hand.

(5) If the centrifuge begins to vibrate strongly and clatter while running, stop it at once and check the balance of the tubes.

(6) Bring it to top speed gradually.

(7) Since others will be sharing the instrument, do your centrifuging efficiently. Get everything ready before occupying the instrument, and get your tubes out of it immediately when the job is finished.

Handling reagents

We have designed the fractionation scheme to keep the use of strong acids and bases to a minimum. You will nevertheless be using normal sulfuric and hydrochloric acids, and barium and sodium hydroxides. Try not to spill, but if you should spill any on the table or floor, clean up immediately, using a fair amount of water to dilute the acid or base. If any spills on you, rinse at once with water, and tell the instructor. Any large spill of acid can be neutralized by sprinkling it with sodium bicarbonate (baking soda).

The alcohol that we use to precipitate glycogen and nucleic acid is flammable, and should not be used close to any flame. If you or your

PROCEDURE FLOW SHEET

To a teaspoonful of washed baker's yeast in a mortar add three times as much sand, and grind thoroughly for 5 min. Add 15 ml of 5% trichloracetic acid, and grind 2 to 3 min more. Let the sand settle, and decant the milky supernatant suspension into a 50-ml plastic tube. Centrifuge 5 min.

PRECIPITATE (nucleic acids, proteins)

Stir this thoroughly into about 15 ml of 10% NaCl solution, and heat in boiling water bath for 10 min. Centrifuge 3 min.

PRECIPITATE (coagulated proteins)

Measure two portions, each about the size of a small pea, on the end of your spatula, into labeled test tubes. To one add about 2 ml of a solution of pancreatic enzymes in phosphate buffer (pH 7.0), and a crystal of thymol to prevent bacterial growth. Swirl the tube to suspend its contents. To the protein in the other tube add an equal volume of buffer alone, and thymol, to serve as control. Label and initial both tubes, and give to your instructor to store until next week.

SUPERNATANT (nucleic acids)

Decant this into another centrifuge tube. Precipitate the nucleic acids by adding 2 volumes of ethyl alcohol, stir, cool several minutes in ice bath. Centrifuge 3 min.

PRECIPITATE (nucleic acids)

Dissolve this in 1 ml of 1 *N* H_2SO_4. Transfer half the solution to a small test tube, and heat 30 to 60 min in a boiling water bath. (If the sample begins to dry out, bring back to volume with distilled water.) Transfer the other half to another small test tube (label and initial both). Neutralize both portions with $Ba(OH)_2$. $BaSO_4$ precipitates; ignore it. Plug the test tubes with rubber stoppers, and give to your instructor to store until next week.

SUPERNATANT (Discard)

SUPERNATANT (glycogen)

Decant the supernatant into another (labeled) centrifuge tube. Precipitate the glycogen by adding 2 volumes ethyl alcohol, stirring, and cooling in an ice bath. Centrifuge 3 min.

PRECIPITATE (glycogen)

Dissolve this in 2 to 3 ml of 1 *N* HCl, and divide evenly between two glass test tubes. Immediately neutralize one half with 1 *N* NaOH. Heat the other half in the boiling water bath for 30 to 60 min. (If it begins to dry out, add distilled water.) Then cool, and neutralize in the same way. Transfer each sample into a dialysis sac with a medicine dropper, and suspend the sacs in labeled and initialed test tubes $\frac{3}{4}$ filled with distilled water. (See demonstration.) Store in refrigerator until next week.

SUPERNATANT (Discard)

neighbors need to have a flame at the bench, either delay the alcohol precipitation or do it at a distance. Locate the fire extinguisher and fire blanket early in the period, in case they may be needed.

Neutralization of acids and bases

You have four neutralizations of acids to perform, two on the hydrolysates of glycogen and nucleic acid, the other two on the controls for these reactions. The glycogen had been taken up in normal (i.e., 1 *N*) HCl, and is neutralized with 1 *N* NaOH. A *normal* solution contains 1 gram molecular weight of hydrogen or hydroxyl ions per liter. The molecular weight of HCl is 36.5 grams, so that a normal solution contains this amount of HCl per liter; similarly a normal solution of NaOH contains 40 grams per liter. (How do we obtain these numbers?) A given volume of 1 *N* HCl should be very nearly neutralized by the addition of the same volume of 1 *N* NaOH. What are the products of this neutralization?

To neutralize the glycogen solution in HCl, add about 30 drops of NaOH solution (about 1.5 ml), stir with a glass rod, and then touch the end of the wet rod to a piece of red litmus paper. Such paper is red when acid, but turns blue on being made alkaline. You will find that the wet spot you have made remains red, as it should at the beginning of such a titration. Now add more NaOH drop by drop, stirring well each time with the glass rod, and touching a fresh bit of the litmus paper each time with the wet rod. Do this until the addition of a final drop just turns the paper blue. That marks the end of your titration. The faint blueness of the paper shows that your solution is now slightly alkaline, which is how we want it.

The same essential procedure is used to neutralize the H_2SO_4 in which nucleic acid was hydrolyzed. The sulfuric acid solution also is 1 *N*. Since each molecule of H_2SO_4 contains 2 atoms of hydrogen, to make a normal solution we dissolve *half* the gram molecular weight of H_2SO_4 (i.e., $\frac{1}{2}$ of 98 = 49 grams) in one liter. In this case we use barium hydroxide to neutralize the acid. The reason is that the salt formed by the neutralization, $BaSO_4$, is insoluble and precipitates, leaving a salt-free solution in which the paper chromatography to be done next week will go better than if the salt were present.

Fractionation and isolation of molecules

One of the principal tasks in biochemistry is to divide a complicated mixture of molecules into its components, ultimately separating out single molecular species. This is the enterprise in which you are now engaged, and we should like to say a little more about the procedures you are using.

The crudest of them involves separating a soluble from an insoluble fraction. That could be done by filtration, the filter paper holding back the insoluble material and permitting the clear solution to run through. We accomplish the same thing more rapidly and cleanly by centrifugation, which among other things avoids losing any material such as inevitably would have stuck to filter paper. This is one of the commonest procedures in biochemistry and can be used to throw down any particles which are denser than the liquid in which they are suspended.

A second method of fractionation in these experiments is *dialysis*. This is a refined kind of filtration, in which the dialysis sac is the filter. It is composed of a cellulose membrane that contains tiny pores which allow water and small molecules to pass through but block the passage of macromolecules. In other words, this process divides small from large molecules. In your dialysis setup, the large molecules should stay inside the sac, and the small molecules distribute themselves evenly inside and outside. If, however, you had chosen to replace the outside solution repeatedly with fresh distilled water, you would eventually have removed all the small molecules, and only the large molecules would have remained inside the sac. In this way you could have *washed* the macro-

molecules inside the sac, freeing them from all contamination with small molecules. Dialysis therefore is one of the most useful procedures, not only for distinguishing large from small molecules, but for purifying large molecules.

Next week we shall work with an ultimate fractionation method, paper chromatography. This can separate individual molecular species, one from another, even when they differ only slightly in structure. It requires also extremely small amounts of material. Its sensitivity and accuracy of resolution make it one of the most useful procedures now available for biochemical analysis.

EQUIPMENT

Per student

4 small test tubes with stoppers
8 test tubes (16 × 150 mm)
4 50-ml plastic centrifuge tubes
3 stirring rods
400-ml (or 250-ml) beaker
bunsen burner
test-tube rack
6″ medicine dropper
2 ft of $\frac{3}{8}$″ dialysis tubing

Per 2 students

mortar and pestle
wax marking pencil

Per 8 students

matches
5% trichloracetic acid (250 ml)
2 vials of indicator paper
2 dropping bottles of distilled water (with glass stopper)
2 dropping bottles of 1-*N* HCl (with glass stopper)
2 dropping bottles of 1-*N* H_2SO_4 (with glass stopper)
2 dropping bottles of 1-*N* NaOH (with rubber stopper)
2 dropping bottles of 1-*N* saturated $Ba(OH)_2$ (with rubber stopper)
dropping bottle of 0.5-*M* glucose (with rubber stopper)
10% NaCl (200 ml)
95% ethanol (500 ml)

Per 30 students

2 clinical centrifuges
pair of scissors
2 ink marking pencils ("Magic Markers")
1 lb of purified sea sand
phosphate buffer (0.1 *M*, pH 7.0) (200 ml)
0.1% pangestin in phosphate buffer (0.1 *M*, pH 7.0) (200 ml)
thymol ($\frac{1}{4}$ lb)

Per laboratory

molecular models (can be purchased from E. H. Sargent & Co., Chicago, Ill.)
water baths at 100°C
refrigeration space for 2 test tubes per student
yeast preparations

Commercial brewer's yeast contains very little glycogen and is therefore unsatisfactory for this experiment. Baker's yeast does contain glycogen, but the cakes in which it is supplied are held together with starch binder. The starch is in the form of grains which are larger and denser than the yeast cells, so that they are easily removed by sedimentation.

Suspend the yeast in water or dilute salt solution, and centrifuge very briefly, for about 15 sec, at 500 to 1000 rpm; or let the suspension stand until the starch grains settle. The upper layer of the suspension can then be decanted and centrifuged to pack the yeast cells. A few repetitions of this procedure should be enough to remove all starch. This point is easily demonstrated by staining one drop on a slide with iodine-KI (Lugol's) solution (see page 24). Starch, of course, stains blue or purple, whereas the yeast glycogen stains reddish brown, entirely within the cells.

Test tubes to be put into the water bath can be labeled with masking tape.

IV CHEMICAL COMPONENTS OF CELLS: MACROMOLECULES OF YEAST AND THEIR SUBUNITS (2)

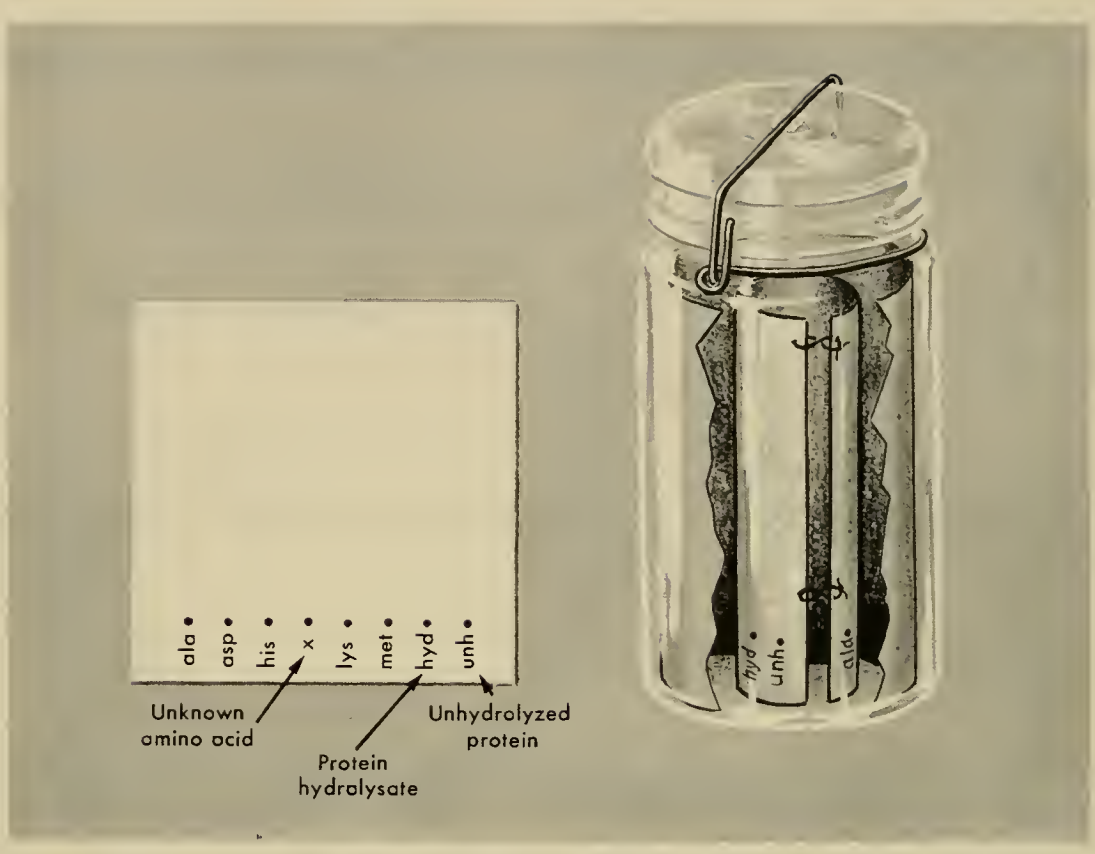
(Reading: W. H. Stein and S. Moore, "The Structure of Proteins," *Sci. Am.* **192**, No. 5, 36–41, May 1955.)

Paper chromatography separates compounds on the basis of their different rates of migration on filter paper (cellulose). The rates of migration depend upon the solvent which is flowing up or down the paper, and on the relative strengths of adsorption which hold the molecules more or less tightly to the paper. Some substances can be separated fairly well in distilled water, but mixtures of water with various organic solvents are usually more effective. Depending upon whether the solvent flows down or up the paper, one speaks of descending and ascending chromatograms. We will use ascending chromatograms.

Chromatography of amino acids and protein hydrolysates

In this experiment you will chromatograph on a single sheet of filter paper your unhydrolyzed protein, the protein hydrolysate you have prepared, an unknown amino acid, and five known amino acids (alanine, aspartic acid, histidine, lysine, and methionine). All these things will be lined up as though getting ready for a race. The measurement will consist in permitting them to run for a time, and then finding how far each has gone. The ratio of the distance a given substance has moved from the starting point to the distance traveled by the solvent front is called the R_F. Two substances having the same R_F, particularly when this has been measured in a variety of solvents, are probably identical; and the R_F of any known substance under particular conditions is an important identifying property.

Lay a square piece of filter paper, 12-cm across, on wax paper, and draw a fine line, with a *lead pencil*, parallel to and 1.5 cm from one edge. This will be the bottom of your chromatogram. (*Note:* Avoid excess handling of the filter paper, since your hands may contaminate it with amino acids. Touch it only at the edges.) On this line mark pencil dots, 13 mm apart, starting 20 mm from one edge. These are to indicate the positions for placing your samples.



Label each sample directly on the paper below the line, as shown on the diagram.

The samples are applied to the paper with a fine glass capillary. Draw a little solution into a capillary, and touch it to the paper at a pencil dot. *Let this dry*, and repeat. Each spot should be not more than 3 mm in diameter. Two such superimposed applications should be enough with the amino acid solutions, and four with the protein and protein hydrolysate solutions. It will be advantageous to place your unknown amino acid in the middle, between the third and fourth known amino acids. Now roll the sheet into a cylinder, and tie the edges together with needle and thread, leaving a gap, as shown in the diagram and as demonstrated by your instructor.

Pour 30 ml of solvent (formic acid:isopropanol:water = 10:70:20) into a quart jar. Line the walls of the jar with a piece of filter paper dipping into the solvent in order to help saturate the atmosphere. Splash the solvent about. Now stand your filter paper cylinder in the jar, keeping it away from the walls, close the jar, and let it stand quietly. Wait until the solvent has risen within 0.5 cm from the top of the paper before removing the cylinder and letting it dry. Cut the threads, dip the paper into the ninhydrin-acetone reagent, and set it aside to dry. After the acetone has evaporated, place the paper in the warm oven (80°) for a few minutes. *Do not overheat!* Remove the paper (you may now handle it), and immediately outline with pencil the spots that you see. (The reason for this is that they fade in the light.)

Make a table showing the R_F values of the known amino acids. Also enter in this table the R_F value of your unknown amino acid. On this basis, what do you think it is? Compare the chromatograms yielded by the unhydrolyzed

protein and the protein hydrolysate. Interpret your results.

Chromatography of nucleic acid components

Your extract of yeast nucleic acids contained both RNA and DNA (see the discussion at the beginning of Exercise III). The hydrolysis that you have performed not only broke the nucleic acid into its component nucleotides, but went on to hydrolyze the nucleotides into their unit components. What we are looking for now, therefore, are the isolated nitrogenous bases. The RNA brought in four such bases: adenine, cytosine, uracil, and guanine; but since guanine is relatively insoluble and difficult to detect on chromatograms, we disregard it in this experiment. Also, since DNA is present in yeast in much smaller amounts than RNA, we shall disregard its distinctive base, thymine.

Prepare a sheet of filter paper, just as before, to run on a single chromatogram your unhydrolyzed and hydrolyzed nucleic acid solutions, a series of three known nitrogenous bases—adenine, cytosine and uracil—and a mixture of the bases. When the paper is ready, put five superimposed applications of each of these solutions at each of the labeled starting positions.

Prepare a second quart jar just as you did the other, but using as solvent acetic acid : butanol : water = 15 : 60 : 25. Set this chromatogram up just as you did the other, stopping it when the solvent has reached 0.5 cm from the top of the paper. Then remove the paper, let it dry, and cut the threads.

Instead of staining the paper this time, as we did to find amino acids, we shall take advantage of the fact that nucleic acids, because of the nitrogenous bases which they contain, strongly absorb ultraviolet light of wavelengths about 260 mμ. After drying your chromatogram, hold it under a source of ultraviolet light. The organic bases will appear as dark spots against the light background. (*Caution:* Do not look into the ultraviolet light. It is harmful to the eyes. Do not expose your skin for more than a few seconds.)

Dialysis of glycogen

Get the test tubes containing the dialyzed samples of your unhydrolyzed and hydrolyzed glycogen from the refrigerator. The point is now to test for glycogen and its subunit, glucose, both inside and outside each of the sacs.

Pour the contents of each bag into a separate clearly labeled test tube so that you now have four solutions: unhydrolyzed inside, unhydrolyzed outside, hydrolyzed inside, hydrolyzed outside. Pour about 1 ml of each of these solutions into a labeled test tube. Into a fifth test tube measure 1 ml of water as a blank. (*Note:* 1 ml = 20 drops.) To each tube now add 2 ml of iodine reagent (iodine-potassium iodide solution), which stains glycogen red-brown. (Do you remember the purple staining of the starch grains in potato slices and *Spirogyra*? That color resulted from the fact that starch contains a straight-chain blue-staining component, in addition to a branched red-staining component. See page 3.)

After you have determined and recorded the fractions in which glycogen is located, wash out the 5 test tubes in which you did the iodine test. Pour a fresh 1-ml sample of each of the 4 fractions and 1 ml of water into the 5 test tubes. To each add 3 ml of Benedict solution, swirl to mix, and place all 5 test tubes in the boiling water bath for 3 min. Now compare the colors.

The Benedict test is given by all sugars that contain reducing groups (aldehyde or ketone) that can reduce blue cupric (Cu^{++}) ions to red, insoluble cuprous (Cu^{+}) ions. The Benedict test is negative for glycogen or starch, because in them the repeating glucose subunits, each of which has a potential aldehyde group (see discussion in III), use up these groups in the glucose-glucose linkages. However, when the glucose units are freed by hydrolysis, the aldehyde groups become available for reaction.

What are your results and what do they mean? The Benedict test is not given by the sugar most familiar to you, cane sugar or sucrose. Why not? Make a model of sucrose.

EQUIPMENT

Per student

test-tube rack

400-ml (or 250-ml) beaker

bunsen burner

ring

wire gauze

2 stirring rods

7 test tubes (16 × 150 mm)

2 quart jars with tops

dropping pipet

Per 8 students

matches

capillary tubing

2 needles and white thread

2 small test tubes of 10^{-3} *M* solutions of each of the following: alanine, aspartic acid, histidine, lysine, and methionine

bottle of Benedict solution (250 ml) prepared as follows:

Dissolve 173.0 of sodium citrate and 100 gm of sodium carbonate in 800 ml of water by heating. Filter if necessary. Dissolve 17.3 gm of copper sulfate in 100 ml of water. Add it slowly to the citrate-carbonate solution, with constant stirring. Make up to 1 liter with water.

2 small test tubes of 1.0 mg/ml solutions of each of the following: adenine, cytosine, uracil, and 1 of a mixture of the three

bottle of Lugol's solution (250 ml) prepared as follows:

Dissolve 1 gm of iodine (I_2) and 2 gm of potassium iodide (KI) in 20 ml of water. Add this to 980 ml of aqueous solution containing 25% KCI (w/v).

Per 30 students

0.5% ninhydrin in acetone (1 liter)

formic acid, isopropanol, water (10:70:20) (2 liters)

acetic acid, butanol, water (15:60:25) (2 liters)

12-cm^2 Whatman No. 1 filter paper (300 sheets)

wax paper (2 rolls)

Per laboratory

ultraviolet germicidal lamp and safety glasses

warm oven

water baths at 100°C

V ENZYMES

(Readings: Weisz, pp. 135–141 and 271–273. S.P.T., pp. 93–96. Villee, pp. 57–65, 306.)

Living cells have the remarkable capacity to perform rapidly and under mild conditions chemical reactions which under the same circumstances would proceed extremely slowly outside the organism. A homely example: sugar exposed to oxygen burns to carbon dioxide and water, generating considerable heat in the process. If you touch a match to the sugar, thus providing energy of activation, this reaction goes very rapidly, as you know. Without the match, i.e., at room temperature, the same reaction goes in exactly the same way, yielding just as much carbon dioxide, water, and heat, but so slowly as to be negligible. In a frog at room temperature, however, or in yourself at a slightly higher temperature, the same reaction occurs rapidly, yielding exactly the same products, and exactly the same amount of energy, though the latter, before being degraded finally to heat, is used for all the multiple activities of the organism.

The enzymes of living cells greatly accelerate such chemical reactions, and by governing their relative rates, regulate the overall directions of metabolic change. Enzymes are *catalysts:* they greatly speed a chemical reaction, without themselves being used up in the process. It is not that they don't take part in the reaction. They do, by combining for a moment with the reactant, the *substrate;* but at the end of the reaction the enzyme is returned and can be used again:

$$\text{enzyme} + \text{substrate} \rightleftharpoons \text{enzyme-substrate complex} \rightleftharpoons \text{enzyme} + \text{products.}$$

This is what we mean by a catalyst; and for this reason a little enzyme goes a long way.

Since the enzyme is returned unchanged at the end of the reaction, it can contribute nothing to the final result. If the reaction is reversible, the presence of the enzyme hastens, but does not change, the final equilibrium. That is, in any reversible system, the enzyme speeds up equally the forward and the back reaction. This behavior also is typical of all catalysts. Thus the pancreatic enzymes you have already used catalyze equally well the hydrolysis and the synthesis of peptide linkages; yet because the equilibrium of this pair of opposed reactions lies far over toward hydrolysis, and because the reaction usually occurs in the presence of overwhelming concentrations of water, an almost irreversible hydrolysis is the end result.

All known enzymes are proteins, and many of their properties depend upon this fact. Their activity depends, as do many other protein

properties, on the hydrogen ion concentration of the medium. Each enzyme tends to be most active over a narrow range of hydrogen ion concentration, the "pH optimum." Enzymes are rapidly destroyed by boiling, as are proteins generally.

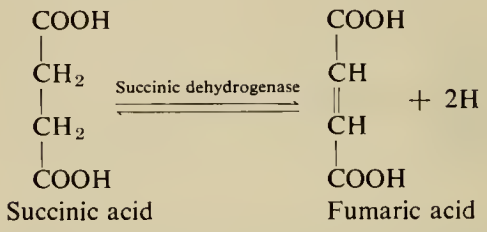
Another general property of enzymes, as of other proteins, is *specificity*. Each enzyme catalyzes only one or a narrow class of chemical reactions. Hence thousands of different enzymes are needed to catalyze the multitude of chemical reactions carried out by living cells.

It is one of the triumphs of modern biochemistry to extract enzymes and enzyme systems from cells and have them catalyze in the test tube the same reactions and reaction sequences that we find in living organisms. Indeed a great number of enzymes have been prepared pure and crystalline, and many are now bought and sold commercially like other organic substances.

In this period we shall work with three enzyme systems, each of which has something special to tell us. *Succinic dehydrogenase* is an oxidation-reduction (hydrogen-transferring) enzyme, of central importance in cellular respiration. With it we can demonstrate hydrogen transfer, and the mechanism of action of a powerful respiratory poison. *Amylase* is a digestive enzyme, which catalyzes an almost irreversible hydrolysis; with this system we can readily measure the effects of changing enzyme concentration, pH, and temperature on the rate of reaction. *Phosphorylase* catalyzes the coming to equilibrium of a reversible system, and so permits us to study synthesis as well as degradation, depending upon how the system is constituted.

SUCCINIC DEHYDROGENASE

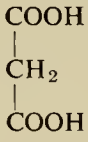
The citric acid or Krebs cycle is central among the enzyme systems concerned with cellular respiration, the process by which organic molecules are burned with molecular oxygen to carbon dioxide, water, and energy in forms useful for cellular work. One of the steps of this cycle is the oxidation of succinic acid to fumaric acid. This reaction is catalyzed by the enzyme succinic dehydrogenase:



Succinic acid Fumaric acid

This reaction can be followed by observing the loss of color of the dye methylene blue (MB) as it is reduced to the colorless form "methylene white" ($MB\text{-}H_2$) by accepting the two hydrogen atoms removed from succinic acid.

We have already spoken of the specificity of enzymes for their substrates. Succinic dehydrogenase, so far as we know, catalyzes only the dehydrogenation of succinic acid, in part because the catalytically active site on the enzyme molecule combines readily with succinic acid to form the enzyme-substrate complex. Sometimes, however, it is possible to fool an enzyme by offering it a molecule that so greatly resembles its normal substrate that the enzyme combines with the impostor instead. Such a molecule in the present instance is malonic acid:



Succinic dehydrogenase, having combined with malonic acid rather than succinic acid, can neither dehydrogenate it nor lose it again. Thus its active site is blocked, and the enzyme is inhibited or poisoned. The inhibition is as specific as the enzyme action and for the same reason. It can be reversed by adding an excess of succinic acid, which competes with malonic acid for the catalytic site. We call malonic acid for this reason a *competitive* inhibitor. Its action on succinic dehydrogenase makes it about as powerful a poison of cellular respiration as cyanide.

Try to find time after doing the experiments to make molecular models of succinic and malonic acids. What resemblance between these molecules do you suppose fools the enzyme?

Experiment

Succinic dehydrogenase occurs in the cell particles known as mitochondria. It can be obtained directly from beef heart. A piece of meat about the size of a small marble should be used for each assay. The meat should be cut up further and washed a few times by vigorous shaking with water in a test tube, followed by decantation of the wash water in order to remove any substrates already present. Add reagents to the labeled tubes as follows, agitating so that added substances are evenly distributed throughout the muscle suspension:

Tube 1: no meat; add an equivalent volume of water plus 3 drops succinic acid (0.5 *M*) and 7 drops methylene blue (MB) solution (0.01%).

Tube 2: meat plus 3 drops succinic acid.

Tube 3: meat plus 7 drops MB.

Tube 4: meat plus 3 drops succinic acid plus 7 drops MB.

Tube 5: meat plus 3 drops succinic acid plus 3 drops malonic acid (1 *M*) plus 7 drops MB.

Tube 6: meat plus 9 drops succinic acid plus 3 drops malonic acid plus 7 drops MB.

Tube 7: boiled (2 min) meat plus 3 drops succinic acid plus 7 drops MB.

Bring the solutions in all tubes to the same total volume (19 drops, as in Tube 6) by adding distilled water.

Pour mineral oil down the side of each tube so as to form a surface layer not more than 1 cm thick. The oil keeps oxygen from diffusing in, and so prevents reoxidation of MB-H_2. Place the tubes in the water bath at 37°C, and watch for color changes while you go on with other experiments.

What changes have you observed? Why was the experiment set up in seven test tubes as above? What does each mixture contribute? Could you have learned as much from fewer mixtures? At the end of the experiment, you can demonstrate the rapid oxidation of reduced methylene blue by air by stoppering Tube 4 with your finger and shaking it violently.

SALIVARY AMYLASE

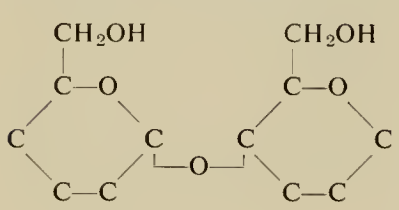
Starches are very large carbohydrate molecules, made by stringing hundreds to thousands of glucose molecules together in long straight and branched chains (Review Exercise III). Saliva, which is secreted by the salivary glands in amounts of the order of 1 liter daily, contains an enzyme that catalyzes the hydrolysis (digestion) of starch through a series of smaller and smaller intermediates (so-called dextrins) to the final product *maltose*, which consists of two glucose molecules joined together as in starch. To hydrolyze maltose to glucose requires another enzyme, maltase, not present in saliva, but secreted by both the pancreas and the small intestine.

The salivary enzyme that hydrolyzes starch to maltose is called salivary amylase. It has been prepared in crystalline condition. Older names for it are salivary diastase and ptyalin.

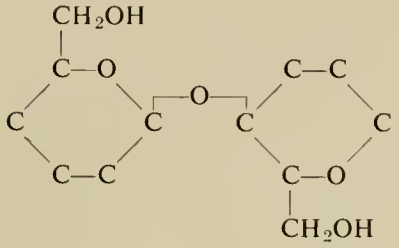
Its action can be followed readily with the *iodine test*. Iodine yields a deep blue color with starch (actually only with the straight-chain, amylose fraction of starch). As the starch is hydrolyzed, repeated tests with iodine go from the initial blue color to red or reddish brown (dextrins), and eventually to colorlessness (smaller dextrins, maltose).

A word about the shapes of molecules and specificity of enzymes. Like starch, cellulose is made of glucose molecules tied together to form very long chains. The only essential difference between starch and cellulose—one of the most biochemically reactive, and one of the most inert molecules—is that in starch the glucose

molecules are bound in so-called alpha-linkage, in cellulose in beta-linkage:



Maltose, with two glucose molecules linked as in starch.



Cellobiose, with two glucose molecules linked as in cellulose.

(Write all the missing —H and —OH groups into the above structures.)

The essential difference between starch and cellulose is therefore one of *molecular shape;* but that is reason enough for the amylases, which digest starch, to have no effect on cellulose, and for the rare group of enzymes that digest cellulose (cellulases) to have no effect on starch.

When you have time, make the molecular models of maltose and cellobiose, starting in each case with two molecules of glucose.

Experiments

Reaction rate vs. enzyme concentration. Stimulate your flow of saliva by chewing a piece of gum, and collect about 5 ml in a test tube. Working with your partner, make a series of dilutions in tap water as follows:

Dilution (ml saliva: ml tap water)	Concentration of saliva (%)
1:9	10
1:19	5
1:49	2
1:99	1

These are conveniently made by taking 1 ml of saliva for each dilution (using a pipet) and making it up to the indicated total by adding tap water from a 50-ml graduate. (Do not use the same pipet for the starch solutions.) Measure the activity of these four dilutions of saliva as follows:

(1) Pipet 1 ml of each concentration of saliva into a test tube and label.

(2) Into each of a second series of four test tubes pipet 2 ml of 0.5% starch suspension, made up in 0.25% NaCl. (Salt is added because chloride ions specifically activate salivary amylase.) Add 2 ml of buffer solution, pH 6.8, to each tube (this is the optimal pH for the enzyme).

(3) Place the two sets of tubes (eight in all) in the water bath at 37°C. Leave for several minutes until they reach that temperature. (*Note:* This experiment can also be performed at room temperature but will go more slowly (see next exercise).

(4) At a recorded time, pour the contents of one tube containing starch mixture into the tube with the highest concentration of saliva. Swirl to mix, and return it quickly to the bath.

(5) Working with your partner, test for starch by removing a drop of the reaction mixture with a medicine dropper and adding it to a drop of an aqueous solution of I_2 in KI (each 0.01 *M*) on a test plate. (*Note:* The I_2-KI solution should not be allowed to stand in the test plate depressions for more than a few minutes. Dispense it one drop at a time as needed.) These tests should be started at a time as near zero as possible, and continued at 10-second intervals thereafter. The initial color should be blue; continue the tests until they yield no color change at all. Note the colors you see, and record the time required to reach the endpoint, the point at which the mixture has the same color as the iodine test solution.

(6) Repeat the procedure of steps 4 and 5 with the 1:19 dilution. Depending on the rate of reaction, the time intervals between tests can be lengthened.

(7) If the activity of the saliva is not too great (if, for example, it takes more than a minute for the 1:19 dilution to reach the endpoint), the remaining two dilutions (1:49 and 1:99) can be run simultaneously to save time, and the test intervals can be increased again to keep pace with the rates of reaction.

Plot a graph showing the reciprocal of the time (1/min) required to reach the endpoint vs. the concentration of saliva. The reciprocal of the time is a measure of the rate of reaction.

Compare the activity of the saliva used in your tests with that used by other students in terms of the times required to reach the achromatic endpoint in the tubes to which 2% saliva was added. Compare the result in your own experiment with the minimum, maximum, and mean values of the class as a whole. What would you conclude?

Reaction rate vs. temperature. (*Note:* Half the students in the class should do this experiment, the other half the experiment on acidity below, in each case working in pairs.) Using the same techniques and the same pH as in the previous experiment, and selecting a saliva concentration that yields an endpoint in 3 to 4 minutes, determine the rate of the reaction at 0°C (ice in water), room temperature, 37°C (water bath), and 100°C (boiling water). At 0° and 100°, tests can be made at intervals of 1, 5, or 10 minutes, after it has become clear that the reaction is going slowly.

Plot a graph of 1/min to endpoint vs. temperature.

Chemical reactions in general go 2 to 3 times faster for every 10° rise in temperature. The same tends to be true of enzyme-catalyzed reactions, with a special twist: as the temperature rises, it reaches a point at which it begins to destroy the enzyme, as it does other proteins, and thereafter the reaction rate *falls* instead of rising further. The result is that as the temperature is raised from some low initial value, the rate of the catalyzed reaction first rises, then falls. At a certain temperature, just before it begins to fall, the rate is at its highest, the so-called *temperature optimum*.

From your observations, about where do you estimate the temperature optimum for salivary amylase to lie? How is it related to your body temperature? If you now brought both the 0° and the 100° samples to 37°, what reaction rates would result? Why?

Reaction rate vs. pH. Determine the time to reach the endpoint at 37° in reaction mixtures buffered at pH 3.4, 5.0, 6.8, and 8.0. Mix 2 ml of the starch-NaCl solution, 2 ml of the appropriate buffer, and 1 ml of a dilution of saliva deemed suitable on the basis of your previous measurements.

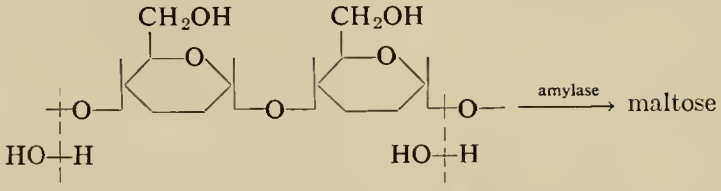
What do you conclude to be the approximate pH optimum of salivary amylase? On what side of neutrality does it lie? How is it related to the pH of your saliva? (Measure this by touching the end of a piece of pHydrion paper to your tongue and comparing with the color scale.)

Whichever of the last two experiments you did, find out what results were obtained in the other experiment, and note them in your laboratory notebook. In general we want you to know everything that goes on in your laboratory, whether you do it yourself or not.

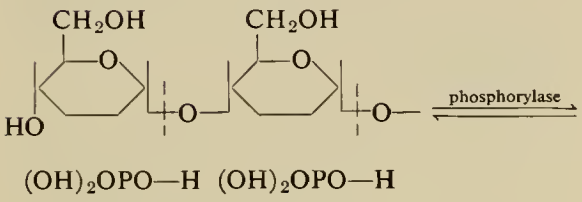
PHOSPHORYLASE

For a long time it was thought that such amylases as you have just examined are responsible for degrading glycogen in animal tissues. Yet liver and muscle degrade glycogen very much more quickly than any known amylases can accomplish. In 1935 a new class of polysaccharide-splitting and -synthesizing enzymes was discovered, called *phosphorylases*. The splitting of glycogen by a phosphorylase requires the presence of inorganic phosphate, and the product is not glucose, but glucose-1-phosphate. Whereas amylases break glycolytic linkages by introducing water (hydrolysis), phosphorylases do the same job by introducing phosphoric acid (phosphorolysis), as shown on the next page.

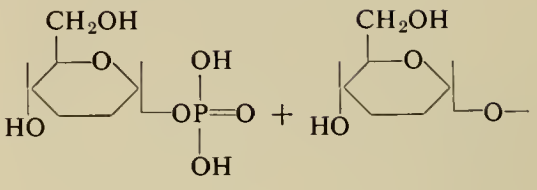
Action of amylase and phosphorylase on polysaccharides:



polysaccharide + water



polysaccharide + phosphoric acid



glucose-1-phosphate + shorter chain

These two ways of degrading polysaccharides differ in fundamental ways. The difference in rate has already been mentioned; the phosphorylases are among the most active of known enzymes. Second, amylases end by cleaving polysaccharides into maltose units, which require a second enzyme, maltase, to yield glucose. The phosphorylases yield instead glucose phosphate units, which are hydrolyzed further to glucose and phosphoric acid by the enzyme phosphatase.

The most interesting and important difference in the action of these enzymes, however, involves the reversibility of the phosphorylase reaction. Whereas the hydrolysis of polysaccharides by amylases is virtually irreversible, their phosphorolysis goes readily in either direction. It is important that you understand the reason for this difference.

Hydrolyses in general tend to be virtually irreversible for two reasons: (1) in polysaccharides, for example, the glucose-glucose bond has an energy of about 3 kcal/mole. The hydrolysis of the polysaccharide results in a loss of this energy; and conversely one should have to add this amount of energy per glucose-glucose link from outside in order to resynthesize a polysaccharide. In the absence of such added energy, only the hydrolysis can occur. (2) Such enzymatic reactions, in and out of the cell, ordinarily occur in the presence of an overwhelming concentration of one of the reactants, water. The molar concentration of pure water is 55.6 *M* (why so?), and most aqueous solutions approach this concentration of water. If we write a reversible equation for a hydrolysis, this enormous concentration of water on one side of the equation pushes the equilibrium very far in the other direction ("mass action effect").

A phosphorolysis presents a very different situation in both regards. On the one hand, the energies of the reactants and products are fairly evenly balanced: a glucose-phosphoric acid bond has very nearly the same energy as a glucose-glucose bond. Hence little energy is lost or need be added in going in either direction. Furthermore the concentrations of reactants and products are more evenly balanced, since here phosphoric acid in relatively low concentration takes the place that water occupies in a hydrolysis. The phosphorylase reaction therefore is freely reversible. In neutral solution and at room temperature the equilibrium lies somewhat over

toward polysaccharide synthesis: the system tends to synthesize rather than degrade polysaccharides.

This synthesis, however, can occur only if two conditions are realized: (1) It proceeds, not from glucose, but from glucose-1-phosphate. The organism must begin by spending considerable energy in forming the initial glucose-phosphoric acid bonds. (2) The synthesis requires the presence of some polysaccharide on which to build ("primer"). The reaction adds glucose units to the end of already existing polysaccharide chains. The phosphorylase reaction builds up and degrades only straight polysaccharide chains. The branching of such chains, or the attack upon branched chains, requires another type of enzyme, which exchanges glucose-1,6-glucose links at branch points for the glucose-1,4-glucose links of straight chains. Unaided phosphorylase, therefore, synthesizes only straight-chain polysaccharides, or straight-chain projections from highly branched polysaccharides. In such reactions the molar concentration of polysaccharide does not change. One starts with the concentration of the primer, and all that happens during the synthesis is that the primer grows bigger as glucose units are added to it:

$$\text{glucose-1-phosphate} + \text{primer} \xrightleftharpoons{\text{phosphorylase}} \text{primer-glucose} + H_3PO_4$$

Phosphorylases occur in many animal and higher plant tissues and in yeast. Today we will extract phosphorylase from the potato tuber. In Exercise I (p. 3) you examined starch grains in potato cells, staining them with the I_2-KI reagent. Try this again today if you like.

Experiment

Prepare the following test tubes:

(1) 3 ml of 0.01 *M* glucose

(2) 3 ml of 0.01 *M* glucose-1-phosphate

(3) 3 ml of 0.01 *M* glucose-1-phosphate

(4) 3 ml of 0.01 *M* glucose-1-phosphate

(5) 3 ml of 0.01 *M* glucose-1-phosphate + 1 ml of 0.2 *M* KH_2PO_4

(6) 3 ml of 0.2% soluble starch + 1 ml of 0.2 *M* KH_2PO_4

(7) 3 ml of 0.2% soluble starch + 1 ml of 0.2 *M* KH_2PO_4

Add a very small drop of the 0.2% starch solution to Tubes 1, 2, 4, and 5 to act as primer. There should be so little starch present in these tubes that the I_2-KI test is negative—check it.

With a paring knife, peel a small potato and cut it into small cubes. Place these in a Waring blendor, add 40 ml of 0.01 *N* sodium fluoride, and grind for 30 sec. (*Note:* FLUORIDE IS A POISON! We use it here to inhibit potato phosphatase, which would otherwise hydrolyze glucose-1-phosphate to glucose and phosphoric acid.)

Filter the homogenate through a double layer of cheesecloth into a beaker. Squeeze out as much of the liquid as you can. Centrifuge the suspension for 3 min, then decant and keep the supernatant. Test this extract to see that it is negative to the I_2-KI reagent. Transfer approximately 10 ml of the extract to a test tube, and heat for 5 min in a boiling water bath.

Now add 3 ml of the enzyme preparation to Tubes 1, 2, 3, 5 and 6; and 3 ml of the boiled enzyme preparation to Tubes 4 and 7. (*Note:* Use the enzyme as soon as you have finished preparing it, since it deteriorates rapidly.)

Test each of these mixtures at once and at 3-min intervals thereafter with I_2-KI. The reaction should be completed within about 30 min. Record and explain your results.

What was the purpose of each component in the mixtures you prepared? What was the point of each mixture? What might have happened had you left the fluoride out of the enzyme preparation? If you have time, try doing that. How do you account for the fact that though the number of polysaccharide molecules has not changed (see above), you now obtain a test with I_2-KI whereas initially you didn't?

EQUIPMENT

Per 2 students

24 test tubes (small or medium); 8 more if possible
8 dropping bottles
1-ml pipet
2 5-ml pipets
50-ml graduate
6 6″ medicine droppers
2 beakers suitable for 0°C and 100°C water baths
potato
porcelain spot plate
cheese cloth (10″ × 20″)

Per 8 students

fresh beef heart
mineral oil
50-ml dropping bottle succinate solution (0.5 *M*, adj. to pH 7.5)
50-ml dropping bottle malonate solution (1 *M*, adj. to pH 7.5)
50-ml dropping bottle 0.01% methylene blue solution
50-ml dropping bottle iodine and potassium iodide solution (0.01 *M*)
50-ml dropping bottle 0.5% starch (boiled) in 25% NaCl
50-ml dropping bottle glucose (0.01 *M*)
50-ml dropping bottle glucose-1-phosphate (0.01 *M*)
50-ml dropping bottle KH_2PO_4 (0.2 *M*)
50-ml dropping bottle 0.2% soluble starch
50-ml dropping bottle sodium fluoride (0.01 *N*)
50-ml dropping bottle McIlvaine buffers (0.1 *M*) at pH 3.4, 5.0, 6.8, and 8.0
vial of pHydrion paper

Per laboratory

water baths at 0°C, 37°C, and 100°C
chewing gum (1 stick per student)
Waring blendor
clinical centrifuge

VI STUDIES IN MICROBIOLOGY (I)*

Bacterial Growth; A Bacterial Enzyme; Comparative Biochemistry

(Readings: R. Y. Stanier, M. Doudoroff, and E. A. Adelberg, *The Microbial World*, Prentice-Hall, 1957, pp. 26–37, 225–239, and 255–256. K. V. Thimann, *Life of Bacteria*, Macmillan, 1955, pp. 3–31 and 550–560. Weisz, pp. 173–175. S.P.T., pp. 484–488. Villee, pp. 131–138.)

Bacteria are single-celled organisms, much smaller than the yeasts, algae, and protozoa, examples of which we have already seen. Many kinds of bacteria can be distinguished on the basis of such characteristics as shape, color, nutritional requirements, and biochemical constitution. We shall first study the bacterium *Serratia marcescens*. It is rodlike in shape, red in color, and requires a source of animal or vegetable material for growth.

As the bacterium takes in nutrient substances from its surroundings and converts them into its own proteins, it grows up to a point at which it splits into two cells, each of which continues to grow in the same manner. That is, it reproduces by fission. We shall follow the growth of *Serratia* by counting the number of cells present before and after letting the bacteria grow for two hours.

In order that growth occur rapidly, the bacteria will be suspended in broth, aerated in order to allow the cells to respire freely, and incubated at 37°C. Furthermore, our source of bacteria will be a young culture, that is, one in which the cells are growing rapidly. In an old culture the cells have used up all the nutrients and are no longer growing. To start growing again when transferred to fresh medium, the cells have to reorganize their machinery, and this results in a delay—a lag period—before growth begins.

The number of cells present in a bacterial suspension can be counted by spreading a dilute sample on the surface of agar (a jellylike material) to which nutrients are added. Each cell grows into a colony, and one counts the colonies.

With the help of the enzymes they contain, microorganisms can carry out numerous chemical reactions. *Serratia marcescens* contains the enzyme catalase which breaks down hydrogen peroxide to water and oxygen ($2H_2O_2 \rightarrow 2H_2O$

*A detailed discussion of preparations for the microbiological experiments in Exercises VI through IX will be found in Appendix A (pp. 135–140).

+ O_2). Catalase is a red iron-porphyrin-protein closely related chemically to the blood pigment hemoglobin. The oxygen which is produced can be measured with a volumeter; thus the enzyme action is easily followed. In particular, we shall study the behavior of the enzyme when different amounts of the substrate (hydrogen peroxide) are added. We shall also investigate the inhibition of catalase activity by hydroxylamine (NH_2OH). This chemical interferes with the enzyme by attaching to and hence blocking the iron atoms upon which its action depends.

Catalase is found in many organisms. We shall study it in bacteria, the horse, and potato plants. In all of them it possesses the same type of enzyme activity, and, as can be judged from inactivation by hydroxylamine, this activity is based on the same active group. (Actually these catalases do vary somewhat in their properties, owing to differences in the amino acid composition of their protein components.) How do you suppose organisms so different from one another come to possess such similar enzymes?

EXPERIMENTS

A note on sterile procedure. In working with bacteria it is necessary to minimize the possibility of stray microorganisms from the air entering the cultures and agar plates. Sterilized glassware and pipets should be used whenever possible. Containers should be opened for as brief a time as possible when material is being transferred. The wire loop which is used to sample a suspension and spread it on agar must be heated in a flame before use. If necessary, dropper pipets and tubes may be sterilized by heating them in a boiling water bath for five minutes. However, always allow an instrument to cool before using it, or else the heat will kill the bacteria with which you are working. Avoid touching pipet tips or the sterile part of cotton plugs with your fingers; also do not place them on the bench.

Bacterial growth

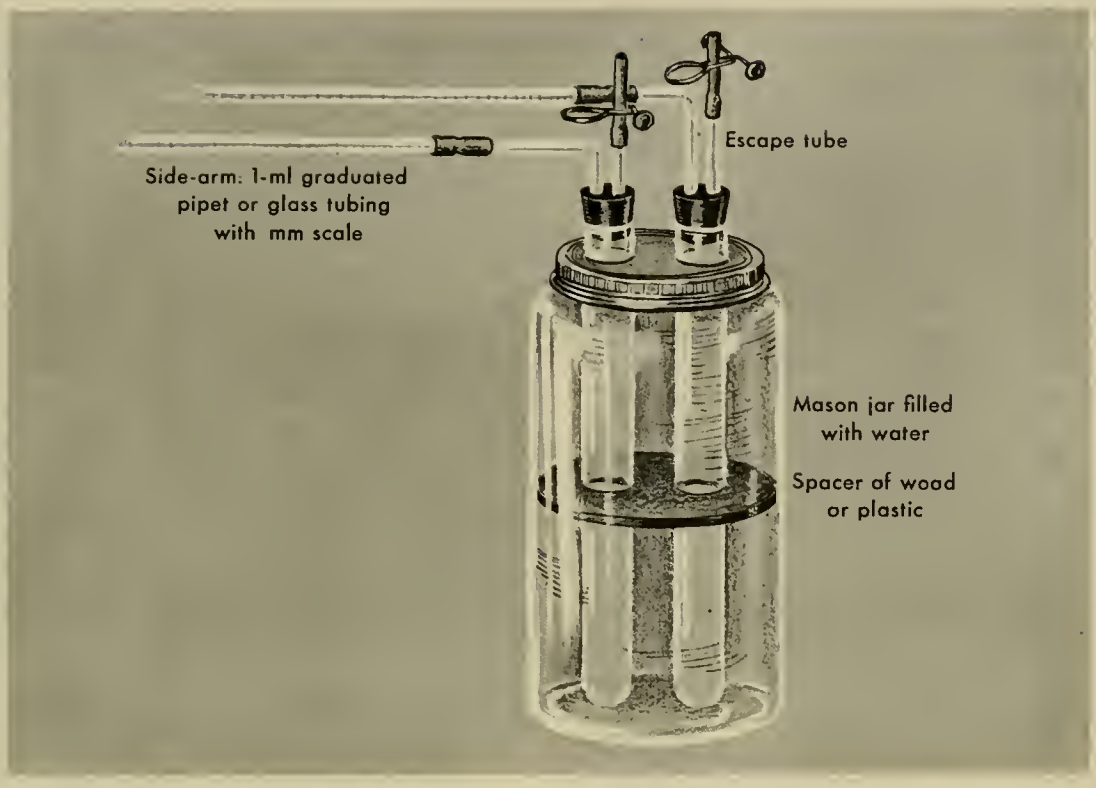
Into a sterile 4-inch test tube pour with sterile precautions 2 ml ($\frac{1}{2}$ inch) of the young *Serratia* culture, and into a wide 6-inch tube pour 20 ml of nutrient broth (about 3 inches). Also obtain nine sterile dropper pipets; wrap these in a clean paper towel before placing them on your bench. With a sterile 5-ml pipet, transfer 5 ml of broth to a wide tube, and without putting down the pipet, deliver 0.9-ml portions of broth to eight 4-inch tubes which will be used for dilutions. With a dropper pipet, inoculate the tube containing 5 ml of broth with 2 drops of *Serratia* culture. Swirl to suspend the bacteria evenly. Label the tube with your name; this will be your culture tube.

Set up a dilution series A with four small tubes, labeled A1, A2, A3, A4. Transfer 2 drops from your culture tube into tube No. 1. Swirl contents to mix, and with a fresh pipet add 2 drops of No. 1 to No. 2. Continue in this manner so that you have a series of four tubes each of which has only $\frac{1}{10}$ as many cells as the one before it. (A drop contains 0.05 ml.) Be careful to add just the right number of drops, since your calculation of the number of cells present depends on the accuracy of the dilutions.

Now, insert a sterile aerator into your culture tube in place of the cotton plug. Attach the long arm of the aerator to the air hose adjacent to the water bath. Adjust the flow of air so that it bubbles gently through your culture. Incubate the tube in the bath at 37°C.

Take an agar plate. Turn the petri dish upside down, and with a wax pencil divide the bottom into four quadrants, labeled A1, A2, etc. Pass a wire loop through the flame, wait a moment for it to cool, then dip it into the No. 4 tube. Make sure you have a loopful of liquid. (A loopful contains just about 0.001 ml.) Gently spread the contents of the loop on the agar in the appropriate quadrant. Repeat with the other tubes. It is not necessary to flame the loop each time when going from lower to higher concentrations. Why?

After your culture has grown for two hours, repeat this procedure to determine the number



of bacteria now present. Label this series B. Take the agar plates home with you. Keep them in a fairly warm spot. Within a few days colonies should appear. Count them. Calculate the number of bacteria in your culture before and after the two hours of growth. (Remember that a drop contains 0.05 and a loop contains 0.001 ml.) How many divisions did each cell undergo in this period? How much time was needed for a cell to duplicate itself?

Bacterial catalase

While your culture is growing, continue with other parts of the experiment. Rinse out the small tubes and dropper pipets which were used for dilution series A. These will now be used in the study of catalase. It is not necessary to use sterile procedure in this part of the experiment.

Obtain 5 ml (1 inch) of the old *Serratia* culture in a 6-inch test tube. The old culture is more convenient for enzyme studies since it contains more cells and hence more enzyme than the young culture. Add 10 drops of this culture to a small tube. Add 10 drops of hydrogen peroxide. Wait a few minutes. Observe. What happens when you dip a glowing splinter into an empty test tube? into the reaction tube? Why?

We shall now measure catalase activity with a "volumeter," a device that measures gas exchanges in terms of changes in the total volume of gas at constant pressure (see illustration). One of the volumeter test tubes will contain the experimental material. The other is left empty, to act as a thermobarometer—a means of correcting for changes of gas volume owing to trivial changes of temperature or barometric pressure in the course of the experiment. Such changes should be equal in both tubes if both contain about the same volumes of gas.

Working in pairs, place 3 drops of the bacterial suspension in one of the volumeter test

tubes. Replace the stopper, making sure that the side-arm, as also that of the thermobarometer, is horizontal. Place a drop of kerosene in the end of each side-arm. Unclamp the escape tube, and with the aid of a medicine dropper inserted into the rubber tubing withdraw enough air to pull this indicator drop back to the proximal end of the scale on the side-arm. When all is in order, add one drop of 3% hydrogen peroxide solution to the test tube containing bacteria through the escape tube. Immediately clamp it shut, and read the positions of the indicator drops in both tubes. From now on reread both tubes every minute, agitating the whole volumeter back and forth for 15 seconds before each reading to hasten the escape of oxygen. When the indicator drop has ceased to move, add a second drop of hydrogen peroxide to the bacterial suspension, and repeat the entire performance. (*Note:* The position of the indicator drop can be moved back to the proximal end of the scale just before adding the second drop of hydrogen peroxide.)

Subtract all changes recorded in the thermobarometer from those measured in the experimental tube. Plot a graph showing the volume of oxygen emitted vs. time. (To turn your measurements of the distance the indicator drop has traveled into gas volumes you must of course measure the internal diameter of the side-arm. Better still, calibrate the volume of the side-arm by injecting known volumes of air through the escape tube with a 1-cc syringe.) Does the evolved gas account for all the oxygen you would expect to obtain from one drop (0.05 ml) of 3% hydrogen peroxide? [The equation for this change is: $H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2}O_2$. Starting with the fraction of a mole of H_2O_2 added, knowing from this the fraction of a mole of oxygen that should be evolved, turn this into a gas volume through the relationship: 1 mole of gas at 273°K (0°C) and 1 atmosphere pressure (as here) occupies 22,400 ml. At room temperature on the absolute scale (T°K), this volume is increased by the factor $T°/273$.]

Add two drops of hydroxylamine solution to the test tube containing the bacteria. After one minute, add two more drops of hydrogen peroxide. Take readings until sure of the result. Now add 10 more drops of hydrogen peroxide, and again follow the reaction. Describe your observations. How does the "noncompetitive" inhibition of catalase by hydroxylamine differ from the competitive inhibition of succinic dehydrogenase by malonate studied in Exercise V?

An experiment in comparative biochemistry

Cut off a $\frac{1}{4}$ inch cube of potato. Mash it up with a glass rod in 1 ml of water in a 4-inch test tube. Set up three 4-inch test tubes containing (1) two drops of *Serratia* culture, (2) two drops of horse blood, (3) two drops of potato extract. Add a drop of hydrogen peroxide to each. Observe. After foaming has subsided add a drop of hydroxylamine to each. Swirl to mix. Add a drop of hydrogen peroxide. Is there a reaction in any of the tubes?

EQUIPMENT

Per student

5-ml pipet, sterile
10 dropper pipets, sterile
test tube, sterile
2 test tubes, nonsterile
2 wide tubes, sterile
aerator assembly, sterile
bacteriological loop
2 nutrient agar plates
bunsen burner
wood splint
marking pencil

Per 8 students

nutrient broth in 250-ml Erlenmeyer flask (20 ml)

young culture of *S. marcescens* in wide tube (20 ml)
old culture of *S. marcescens* in 250-ml Erlenmeyer flask (50 ml)
4 dropping bottles of 3% hydrogen peroxide
2 dropping bottles of 10% hydroxylamine, neutralized
dropping bottle of horse blood diluted 1:20 in 0.85% sodium chloride
small potato
paper towels
4 volumeters

Per laboratory

water baths at 37°C with rack accommodating wide tube
provision for aeration at water baths

VII STUDIES IN MICROBIOLOGY (2)

*Bacterial Mutation; Resistance to Antibiotics; Radiation Effects; Action of Lysozyme; Bacterial Anatomy**

(Reading: H. J. Muller, "Radiation and Human Mutation," *Sci. Am.* **193**, No. 5, Nov. 1955, Reprint No. 29. Further readings are suggested at the end of this exercise.)

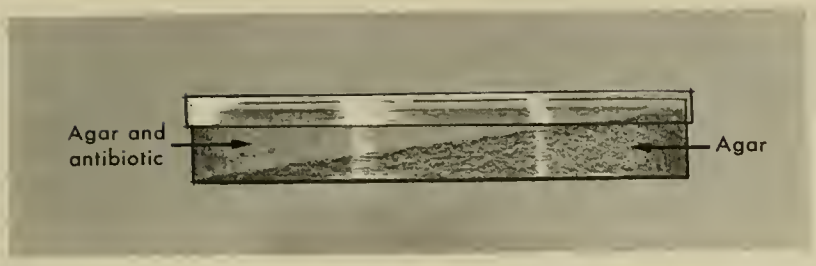
A bacterial population, even though it may have descended from a single cell, contains many cells which differ from the original bacterium and from most of the cells about them. These variants, or mutants, arise spontaneously as the result of aberrations in the molecules concerned with transmitting inheritance from parent to daughter cells, the deoxyribose nucleic acids (DNA). The aberrations responsible for mutation are believed to involve the substitution of one or more nucleotides for others originally present in the DNA sequences. Such errors in the replication of DNA probably occur while these molecules are being multiplied prior to cell division; but whenever they occur, they are propagated thereafter from generation to generation. In this way what begins as a small molecular change can end in forming a new population, a new strain of bacteria.

Even though such changes are rare, there are many of them in a large population. If one in ten thousand bacteria is a mutant, a bacterial population of ten million is likely to have one thousand such mutants. This therefore constitutes a tremendous potentiality for variation, present in all bacterial cultures.

Most mutations are disadvantageous, and thus most mutant strains tend to die out rather than to propagate and expand. A change in environmental conditions, however, may favor a previously unsuccessful mutant. Indeed, a drastic change may kill all the other bacteria and allow one mutant form alone to survive, and—since it is now relieved from competition—to flourish. This is exactly what happens when, after you have taken heavy doses of an antibiotic, you may find that the antibiotic no longer works.

The experiment to be performed today should show you that it is not difficult to develop strains of bacteria that are resistant to penicillin

*Directions for setting up these experiments will be found in Appendix A.



or streptomycin. Some of you may find strains that are resistant to both antibiotics. Such strains do not develop by the reorganization of cells of the original parent stock. On the contrary, the antibiotic eliminates these, and permits only one or a few antibiotic-resistant mutant forms that happen to be present to multiply to form a new antibiotic-resistant population. What we observe, therefore, is not the inheritance of an *acquired* character, but, as always, the *selection* of those individuals which through mutation already possess that character.

As you must know very well by now from discussions of atom bombs and fallout, it is possible to increase the rate of mutation far above that which occurs naturally. One way is by exposing cells to high-energy radiation. Ultraviolet light of wavelengths near 260 mμ has this effect. It is high in energy, corresponding with its short wavelength ($E = hc/\lambda$, in which E is the energy per quantum, h is Planck's constant, c is the velocity of light, and λ is the wavelength). The organic bases in the nucleic acid chains strongly absorb these wavelengths of ultraviolet light. Indeed, such ultraviolet light in large enough doses kills all living cells through its destructive effects upon their nucleic acids. If one subjects a population of cells to a large enough dose of ultraviolet light to kill many of them but not all, the survivors usually display an extraordinarily high incidence of mutation.

Today you will perform such an experiment upon *Serratia marcescens* and look for induced mutants among the surviving cells. The brilliant red pigmentation of this bacterium makes it particularly suitable for such studies, since mutants that lack the normal pigmentation and hence look pink, white, or speckled are easily recognized.

Isolation of antibiotic-resistant strains

Prepare your own agar plate containing an antibiotic concentration gradient by the following procedure (see diagram): Pour enough melted nutrient agar (about 15 ml) into a slanted petri dish (place a stirring rod under one end) so that you have just covered the bottom of the dish. Let the agar harden. Place the plate in a horizontal position and add enough additional agar containing penicillin (P) or streptomycin (S) (whichever one you choose, your neighbor should use the other) to just cover the already solidified agar. (Don't fill the dish to the top.) The antibiotic will establish a linear concentration gradient during subsequent incubation by diffusing into the nutrient agar below it. Before inoculation, dry the surface of the agar by opening the dish slightly by propping up one edge of the lid and incubating the dish for one hour at 37°C. Mark with an arrow on the bottom of the dish the direction of the gradient of antibiotic concentration.

The plate is inoculated with either *S. marcescens* or *Escherichia coli* bacterial suspension, parallel to the gradient. Give the labeled plates to your instructor to store until next week.

Radiation effects

With sterile technique pour about 1 ml of the diluted saline suspension of *S. marcescens* (10^4 cells/ml) into a sterile test tube. Obtain an agar plate containing synthetic medium and divide it into four quadrants marked on the bottom of the glass with wax pencil. Label the quadrants 150, 120, 90 (standing for seconds of radiation),

and control. Spread a loop of the bacterial suspension on the quadrant marked 150 and expose the uncovered plate to ultraviolet light at a distance of 25 inches for 30 seconds. (*Caution:* Do not look into the ultraviolet light. Do not expose your skin for more than a few seconds.) Next spread a loop of the suspension on the quadrant marked 120 and expose the plate to ultraviolet light for an additional 30 seconds. Now spread a third loop on the quadrant marked 90 seconds and expose the plate for 90 seconds. Finally spread a loop of the suspension on the control quadrant, cover, and give the labeled plates to your instructor. He will incubate them for two days at room temperature and will then store them in the refrigerator until next week.

Microscopic examination of *Bacillus megatherium*

Bacillus megatherium is a giant among bacteria even though it is only 1 micron wide by 4 micra long. (A micron is 1/1000 mm, or 1/25,000 inch.) The other strains which we have been using are much smaller, and in order to see them more elaborate microscopy is needed.

Prepare a wet mount of *B. megatherium* as follows. Place a droplet of water on a clean glass slide. This may be done conveniently with a glass rod. Stir a loopful of *B. megatherium* culture into the drop. Gently place a cover glass over the drop; try to avoid leaving air bubbles under the glass. Focus on the bacteria under the high-power objective of your microscope. To do this, watching from the side, bring the objective down until it *just fails* to touch the cover glass. Now, looking through the ocular, slowly raise the objective by means of the fine adjustment until the field is in focus. To see the bacteria well it will be necessary to close down the diaphragm (with the lever under the stage) so that the field is only dimly illuminated.

The bacteria may be seen more easily after staining them with a dye. Remove the cover glass. Let the suspension dry. Pass the slide, face up, through a bunsen flame three times. The heat will coagulate bacterial proteins and fix the bacteria to the slide. When it is cool, flood the area with a drop of methylene blue. Wait one minute, then rinse the slide with water. Gently blot it dry with a paper towel. Examine the slide once again under the high power. Draw what you see.

Action of lysozyme: bacterial protoplasts

The enzyme lysozyme breaks down the complex polysaccharides of which the cell walls of many bacteria are composed, leaving the cell covered only by its delicate plasma membrane. Whereas the intact bacterium may have been rod-shaped, it becomes spherical on losing its rigid cell wall. Such naked, spherical cells are called *protoplasts*. We shall watch cells of *B. megatherium* being lysed by lysozyme, and forming protoplasts. This experiment does not demand sterile conditions.

Quite frequently, as in the present instance, the contents of a cell are considerably more concentrated than the surrounding medium. As a result, water tends to flow from the medium into the cell, making it swell. In *B. megatherium*, as in many other bacteria, this tendency to swell is resisted by the rigid cell wall. (Recall the opposite effect of suspending *Elodea* in strong salt solution, in Exercise I, which made the cell shrink away from the cell wall.) When bacterial cells have lost their cell walls through the action of lysozyme, this restraint is removed. The entrance of water from the medium, swelling the cell, subjects its plasma membrane to great strain. Eventually it ruptures, and the cell contents pour out into the medium.

This is easily seen by adding lysozyme to a turbid suspension of bacteria. The suspension rapidly clears as the cell walls are hydrolyzed away, and the bacteria burst or lyse. If sucrose is added to the medium, so that its osmotic concentration is equal to that of the cell interior, the cell no longer swells, and the result is a stable, spherical protoplast.

Two suspensions of *B. megatherium* are provided, identical except that one is suspended in

dilute phosphate buffer alone (pH 7.0) and the other in phosphate buffer to which sucrose has been added to a concentration of 0.15 *M*, making the medium isosmotic with the cell contents.

Pour about 2 ml of the suspension of cells in buffer alone into a small test tube. Note the turbidity of the suspension. Add 4 to 5 drops of lysozyme solution and swirl, watching the tube as you do so. You should soon see the suspension clarify, as the cells lyse. Examine the end result under the microscope.

Repeat this experiment, using the suspension of cells containing sucrose. Do you still note changes in turbidity? Again look at the result under the microscope. The spherical protoplasts should be visible.

It will be worth preparing a wet mount of *B. megatherium* in phosphate buffer containing sucrose, and adding one drop of lysozyme on the slide while looking at the cells. The dissolution of the cell wall can be seen, and all the stages in the formation of protoplasts.

Microorganisms in the air

Label a plate of nutrient agar with your name, and leave it open, exposed to the air, for 30 minutes. Don't place it too close to where anyone is working, lest he spill bacteria near your plate. These plates should be incubated at home for two days at room temperature (about 20° to 25°C), then placed in the refrigerator so that you can examine them next week.

Further microscopy of bacteria

The microscopes you have been using do not have sufficient magnification to make most bacteria visible. A few higher-power microscopes may be available, possessing an oil immersion objective lens. Since in this case the light is not required to pass from glass to air and back, greater magnification can be achieved. In addition, this microscope may provide phase contrast, which enhances the contrast wherever there is a difference in refraction of light within the object or between it and its surroundings.

Examine wet mounts of *Serratia marcescens* and *Pneumococcus* under the highest power available to you, using phase contrast if you have it. After placing the slide on the stage, put a drop of immersion oil on the center of the cover glass. Watching from the side, bring the high-power objective down until it dips into the oil and almost touches the cover glass. With the fine adjustment slowly *raise* the objective until the bacteria come into focus.

Bacteria are often divided into two groups on the basis of shape: bacilli (rods) and cocci (spheroids). *B. megatherium* is clearly a rod. *Serratia* is more difficult to classify; it is considered to be a short rod. *Pneumococcus* is, of course, considered to be a coccus. Do you find its shape to be perfectly round? Cocci that are strung along in chains are called streptococci (*strepto*, Gr. = chain); those which occur in pairs are diplococci. What would you call *Pneumococcus?*

Further reading

On genes and enzymes:

K. V. Thimann, *Life of Bacteria*, Macmillan, 1955, pp. 561–571.

On radiation and mutation:

R. Y. Stanier, M. Doudoroff, and E. A. Adelberg, *The Microbial World*, Prentice-Hall, 1957, pp. 264–268.

K. V. Thimann, *op. cit.*, pp. 662-667.

On mutation:

S. P. T., pp. 321–324.

R. Y. Stanier, *et al.*, *op. cit.*, pp. 380–393.

On bacteria under the microscope:

R. Y. Stanier, *et al.*, *op. cit.*, Chapter 1 and pp. 105–109.

K. V. Thimann, *op. cit.*, pp. 38–57.

Villee, pp. 132–143.

On microorganisms in the air:

R. Y. Stanier, *et al.*, *op. cit.*, Chapter 5 (fungi); pp. 243–248 (colonial forms); pp. 296–329 (major groups of bacteria).

EQUIPMENT

Per student

petri plate, sterile

glass rod

test tube, sterile

agar plate (synthetic medium)

bunsen burner

2.3% nutrient agar (15 ml)

2.3% nutrient agar plus 2500 units/ml streptomycin (7.5 ml)

2.3% nutrient agar plus 2500 units/ml penicillin (7.5 ml) (the above three solutions all kept at 60°C in water bath)

E. coli: aerating culture (1 ml)

S. marcescens: aerating culture (1 ml)

S. marcescens: diluted saline suspension (10^4 cells/ml) (1 ml)

B. megatherium: about 10 mg/ml dry weight in 0.03 *M* phosphate buffer, pH 7.0 (2 ml)

B. megatherium: as above but in phosphate buffer containing 0.15 *M* sucrose (2 ml)

lysozyme: 5 mg/ml in 0.03 *M* phosphate buffer, pH 7.0 (0.5 ml)

Per 8 students

marking pencils

bacteriological loops

slides and cover slips

water in dropping bottle

methylene blue solution in dropping bottle

Per laboratory

Ultraviolet germicidal lamp and safety glasses

Demonstration slides of *S. marcescens* and *Pneumococcus* under phase contrast microscopes

VIII STUDIES IN MICROBIOLOGY (3)

Genetic Transformation of Bacteria *

(Readings: F. H. C. Crick, "The Structure of the Heriditary Material," *Sci. Am.* **191**, No. 4, 54–61, Oct. 1954, Reprint No. 5. R. D. Hotchkiss and E. Weiss, "Transformed Bacteria," *Sci. Am.* **195**, No. 5, 48–53, Nov. 1956, Reprint No. 18. Further readings are suggested at the end of the exercise.)

One of the most striking characteristics of living organisms is that offspring resemble their parents. This resemblance with regard to both form and function is found in all forms of life from bacteria to man. We tend to take it for granted that human children, like their parents, have five-fingered hands, and three-color vision, yet these traits must just as surely be inherited as such abnormalities as six fingers or color-blindness. In bacteria, heredity operates equally, so that in *Serratia marcescens*, for example, daughter cells, like their parent, are rod-shaped and capable of splitting hydrogen peroxide in a reaction catalyzed by the enzyme catalase.

As you know, the factors responsible for heredity are called genes. All cells contain deoxyribose nucleic acid or DNA, specifically in the nucleus when they have nuclei. That the genes are DNA molecules or portions of them is demonstrated by bacterial transformation: DNA isolated from one bacterial strain can change the nature of a cell of another different strain in ways that are thereafter inherited by all its offspring.

We shall study the transformation of cells of a strain of *Pneumococcus* which is sensitive to the antibiotic streptomycin, by DNA taken from a strain of *Pneumococcus* resistant to this antibiotic. First we shall extract the DNA from cells of the resistant strain by adding sodium deoxycholate, which disintegrates the membranes of the cells, releasing their contents. Next we shall precipitate the DNA by adding alcohol (as in the yeast analysis in Exercise III), so that the molecules form long fibers which can be removed. After redissolving the DNA, this solution will be used to treat bacteria of the sensitive strain which are in an appropriate condition to take up the large molecules of DNA. After allowing the freshly transformed cells to develop resistance to streptomycin, we shall test their ability to form colonies of resistant offspring.

Note on bacterial media and ecology. It may be of interest to you at this stage to learn more about the media used for growing bacteria.

*Directions for setting up these experiments will be found in Appendix A.

Certain species, such as *S. marcescens* and *E. coli*, are very versatile. They can grow on a simple mixture of a sugar and the salts potassium phosphate, ammonium sulfate, calcium chloride, magnesium chloride, and ferric chloride. They do not require vitamins and amino acids, which they can synthesize for themselves. In our experiments, however, we want them to grow more rapidly since we have only a short time in which to work, so we provide them with a richer medium. The nutrient broth we use is a mixture of a protein hydrolysate (such as you made from yeast proteins) and a beef extract, which like whole beef contains salts, vitamins, and sugars as well as amino acids. By adding agar, we obtain a solid growth medium, the surface of which can be used for bacterial counts.

Pneumococcus is a more fastidious organism, and requires many preformed vitamins and amino acids. The growth of *Pneumococcus* can be supported on a medium composed of potassium phosphate, calcium chloride, a protein hydrolysate fortified by the addition of the amino acids cysteine and glutamine, and a tiny trace of yeast extract which acts as a source of vitamins. In order for the cells to become able to incorporate DNA and be transformed, it is necessary to supplement the medium. In particular serum albumin, a protein found in blood, must be added.

In preparing a *solid* medium on whose surface *Pneumococcus* can grow, whole blood must be added. Pneumococcal cells not only do not use air, but are inhibited by its presence. They are so-called *obligate anaerobes*, as opposed to the aerobic bacteria which tolerate the presence of air. What probably happens is that in the presence of oxygen, bacteria produce hydrogen peroxide, which poisons *Pneumococcus* since it lacks catalase. The addition of blood, which contains an active catalase, repairs this deficiency.

The strain of *Pneumococcus* which we use, though not pathogenic, that is, capable of causing disease, is closely related to the strain which causes pneumonia. The chief difference between the two is that the pathogenic strain is covered by a capsule of polysaccharide which protects it in the body. We see that the nutritional and environmental requirements of *Pneumococcus* stem from its parasitic mode of life and the nature of its habitat in body tissues. Another bacterium of wide distribution and interest, *E. coli*, does not grow within the body tissues, but normally is found in the large intestine, where it thrives on the organic material passed along by the digestive apparatus. For this reason it occurs also in sewage and polluted waters.

EXPERIMENTS

Cells of a streptomycin-resistant strain of *Pneumococcus* were grown overnight, collected by centrifugation, and resuspended in the flasks marked SR. Take 5 ml (about $\frac{3}{4}$ inch) of this culture in a wide test tube. Add 5 drops of deoxycholate solution. Mix. Incubate at 37°C for 5 minutes. Do the cells lyse? Does the solution become viscous? (Viscosity of the solution can be estimated by swirling the contents and observing the rate with which bubbles rise.) The increased viscosity is caused by release of the long chains of DNA.

Pour an equal volume of alcohol slowly down the side of the test tube so that it does not mix, but forms a layer over the solution. Gently insert a glass rod into the center of the tube and by rotating the rod wind up on it the fibers of DNA which form at the interface of the alcohol and water. Keep turning the rod until the two layers have mixed. Withdraw the rod with the fibers wound on its end, dip into 2 ml of sterile sodium chloride solution in a small test tube, and stir to remove the DNA. Plug the tube immediately. Swirl until the fibers have dissolved.

Prepare two small test tubes for the transformation experiment by adding 1 ml of medium to each with a sterile pipet. Add to each tube 2 drops of the streptomycin-sensitive cells which are to be transformed. (These will be found in the ice baths. Use a sterile dropper pipet for

the transfer.) Tube No. 1 will be the control. Tube No. 2 should receive 2 drops of your DNA solution. Label the tubes so that you can identify them as your own. Incubate them for 30 minutes in a 30°C water bath. When the 30 minutes are up, transfer the tubes to the 37°C water bath and incubate them for 90 minutes more.

So that you can count the bacteria which are resistant to streptomycin, a blood agar plate containing streptomycin will be provided. Divide the plate into three equal sectors. On one sector spread two loopfuls of culture from the control tube; on another spread two loopfuls of the transformed culture; on the third spread two loopfuls of your DNA solution. Label the sectors appropriately and put your name on the plate. Place it, upside-down, in the bin that is provided. It will be incubated at 37° for two days, and then refrigerated until the next laboratory session.

Antibiotic-resistance experiment (continuation)

Today you will demonstrate that the bacterial colonies which grew on your antibiotic gradient plates are, indeed, mutations. You will test this by transferring mutants from your antibiotic gradient to fresh antibiotic plates. Obtain a nutrient agar plate containing the antibiotic you used last week. The antibiotic concentration is set so that the original strain of *E. coli* will not grow but the resistant mutant will. Divide the plate into four quadrants and label them Hi-P, Lo-P, Hi-S, Lo-S.

Pick a mutant from the end of your gradient plate containing a low antibiotic concentration, and with a wire loop transfer it to a small test tube containing sterile saline and stir. If your antibiotic is penicillin (or streptomycin), transfer a loop of this saline suspension to the "Lo-P" ("Lo-S") quadrant of your new penicillin (streptomycin) plate. Transfer another loopful to the "Lo-P" ("Lo-S") quadrant of your neighbor's streptomycin (penicillin) plate. If any of your bacteria survive when transferred to your neighbor's plate, they should represent double mutants, that is, mutants able to resist both antibiotics. Repeat the operation with a mutant from the high-antibiotic-concentration end of your gradient plate. This mutant should have a better chance of growing on your new plate than the mutant from the low end of the gradient. Should a penicillin mutant have an increased chance of surviving on streptomycin?

Take your plates home and keep them in a warm place for three days. If you find any colonies which you believe to be double mutants, and wish to check them, place your plates in a refrigerator until the next laboratory session (there may be space in the laboratory refrigerator).

Irradiation of *S. marcescens* (continuation)

You can now examine the results of last week's irradiation experiment. Make note of two things: survival, and the presence of color mutants. The latter will be propagated on fresh medium to see if they breed true.

Obtain your irradiated *S. marcescens* plate and pick out a colony that appears clearly to represent a color mutation. With a loop, transfer this colony to a small test tube containing sterile saline. Next, transfer a loop of this saline suspension of bacteria to one quadrant of a new plate (synthetic medium). Repeat this process with three additional mutant colonies. Take your plates home and incubate them at room temperature. A genuine mutation will breed true: the new colonies that result should be identical in color with the original mutant.

Microorganisms in the air (continuation)

While your cultures are incubating, examine the growth on the agar plates you exposed last week to the air. Make a list of the different colonies on the plates; describe them as you see them. Can you distinguish molds from bacteria? Do any two colonies appear to correspond to the same organism?

Examine the different growths under the low power of the microscope. (You may place the petri dish right on the stage.) Record your observations.

Mold colonies are quite beautiful under low power. It should be possible to see numerous strands, or mycelia, which weave into the agar, as well as delicate stalks which lift high black sacs of spores. (*Note:* If there are sporulating molds on the plate, please refrain from agitating them, lest the spores spread into the room and contaminate the blood agar plates.)

Make wet mounts of some of the bacteria and examine them under the high power of your microscope. Can you see cells? What shapes do they have? Are they motile? Record any other interesting observations.

Further reading

On the nature of the genetic material:

A. E. Mirsky, "The Chemistry of Heredity," *Sci. Am.* **188**, No. 2, 47–57, Feb. 1953, Reprint No. 28.

F. H. C. Crick, "Nucleic Acids," *Sci. Am.* **197**, No. 3. pp. 188–200 Sept. 1957, Reprint No. 54.

N. H. Horowitz, "The Gene," *Sci. Am.* **195**, No. 4, 78–90, Oct. 1956, Reprint No. 17.

R. Y. Stanier, M. Doudoroff, and E. A. Adelberg, *The Microbial World*, Prentice-Hall, 1957, pp. 393–401.

On genetic transformation:

E. L. Woolman and F. Jacob, "Sexuality in Bacteria," *Sci. Am.* **195**, No. 1, 109–118, July 1956, Reprint No. 50.

R. Y. Stanier, *et al.*, *op. cit.*, pp. 393–401.

K. V. Thimann, *Life of Bacteria*, Macmillan, 1955, pp. 575–576.

On culture conditions:

R. Y. Stanier, *et al.*, *op. cit.*, pp. 42–45; 48–49.

K. V. Thimann, *op. cit.*, pp. 132–154.

On the ecology of microorganisms and diseases:

R. Y. Stanier, *et al.*, *op. cit.*, pp. 417–573.

On antibiotics:

R. Y. Stanier, *et al.*, *op. cit.*, pp. 257–258.

K. V. Thimann, *op. cit.*, pp. 682–685.

EQUIPMENT

Per student

5-ml pipet, sterile

2 dropper pipets, sterile

wide tube, sterile

2 small tubes, sterile

6 small tubes containing 0.85% sodium chloride solution, sterile

blood agar plate with streptomycin

glass rod

bacteriological loop

bunsen burner

compound microscope

slide and cover slip

agar plate containing synthetic medium

nutrient agar plate (half with penicillin, half with streptomycin; 1000 units/ml is a convenient concentration of each)

Per 8 students

cultures of cells of streptomycin-resistant *Pneumococcus* (45 ml)

cultures of cells of streptomycin-sensitive *Pneumococcus*, competent for transformation, in small tube immersed in beaker of ice (3 ml)

Pneumococcus medium with glucose added, in wide tube (20 ml)

dropping bottle of 5% deoxycholic acid, neutralized alcohol, in reagent bottle (100 ml)

Per 30 students

3 or 4 water baths at 37°C

1 water bath at 30°C

bin for incubating agar plates

IX STUDIES IN MICROBIOLOGY (4)

Viruses: Their Identification, Mode of Reproduction, and Filterability *

(Readings: Weisz, pp. 32–34. S.P.T., pp. 43, 316. Villee, pp. 138–141. F. M. Burnet, "Viruses," *Sci. Am.* **184,** No. 5, 43–51, May 1951, Reprint No. 2. G. S. Stent, "The Multiplication of Bacterial Viruses," *Sci. Am.* **188,** No. 5, 36–39, May 1953, Reprint No. 40. Other readings listed at the end.)

Viruses are particles, smaller than most cells, composed of protein and nucleic acid. Although they are unable to grow or carry out any of the processes characteristic of living things by themselves, they have the curious ability to divert the machinery of a cell so that in place of its normal activity it begins to mass-produce the virus. Some of the most dread diseases of man, smallpox, polio, and rabies, are caused by viruses.

Bacteriophages, viruses which infect bacterial cells, have been intensely studied in recent years and much has been learned of their structure and mode of operation. As an example we shall take the phage called T_4 which attacks cells of the bacterium *E. coli.* Observations made with the electron microscope show the virus to consist of a polyhedral body containing DNA, to which a tubelike structure is appended. It looks like a bulb bearing a tube. The virus attaches to the cell by the end of the tube, and injects its DNA into the cell through the tube. For about 10 minutes, though in this interval viral DNA and protein constituents begin to accumulate, no new virus is formed. Then, during the next 20 minutes, more and more virus particles form until, about a half-hour after infection, the cell bursts and releases over 100 new virus particles.

We shall follow such a growth cycle. Virus particles can be counted by spreading a suspension of them on an agar surface which is covered with a dense population of susceptible bacteria. The bacteria grow except in the areas surrounding each virus particle, where they have been killed by the multiplying virus. Such blank areas, or *plaques*, can be counted in the same manner as bacterial colonies, and from such counts the density of infective virus particles in the original suspension can be calculated.

Under proper conditions, a given phage produces plaques of quite uniform and reproducible morphology. An experimenter can often decide with which bacteriophage he is dealing from the character of the plaques, just as one can often identify a bacterium from the character of its colonies. In the second part of this exercise you will be given samples of three known phages

*Directions for setting up these experiments are in Appendix A.

(T_2, T_4, and T_{4r}), along with one unlabeled sample of one of these three phages. We will plate out all four samples, and by examining the plaque types identify the unknown phage.

One of the most characteristic features of viruses is their small size. This was appreciated very early when it was observed that they pass through filters which have pores fine enough to retain bacteria. For this reason these minute infective agents were called "filterable" viruses. We shall test the filterability of viruses and bacteria with a porcelain filter.

EXPERIMENTS

Bacterial transformation (continuation)

Examine the blood agar plate from last week's experiment on the genetic transformation of *Pneumococcus*. Colonies of *Pneumococcus* have a characteristic appearance on blood agar plates, so they can easily be distinguished from contaminants. The *Pneumococcus* colony is very small, a fraction of a millimeter in diameter. Around the colony is a zone of hemolysis, a clear area where substances released by the cells have lysed the blood cells in the agar. Any colonies which you find on the plates are streptomycin-resistant, since streptomycin had been added to the agar.

Hold the plate up to the light. Do you find any colonies of *Pneumococcus* in the control or DNA sectors? in the sector corresponding to the transformed culture? Count the number of resistant colonies.

Reproduction of bacteriophage

In sterile, wide test tubes obtain 5 ml of nutrient broth. One student should prepare a dilution series of the phage for himself and his partner as follows. Transfer 1-drop portions of the *E. coli* culture to each of 8 small tubes containing 1 ml of soft agar (4 per student). (The soft agar is kept in the water bath at 45°C.) With a sterile dropper, add 1 drop of the phage suspension to the nutrient broth. Mix. Now prepare to determine the number of virus particles, by making a dilution series of the phage in broth in the 4 tubes containing bacteria in soft agar. With the sterile dropper add 2 drops of the phage in broth to the first tube, 2 drops of that to the second, and so on. (Do not let the soft agar harden; keep the tubes in the 45° bath as much as possible during these transfers.)

Obtain a 4-quadrant nutrient agar plate, and label appropriately. Now quickly pour the contents of the dilution tubes onto the appropriate quadrants, one at a time. Rock the plate slightly each time to obtain a thin, even layer of liquid over the quadrant surface, and let harden. Be careful not to spill over onto the neighboring quadrants.

We shall now repeat this experiment after allowing the virus a period of growth. Add 3 drops of *E. coli* cells to the phage suspension in broth. Insert an aerator tube. Incubate at 37° for 60 minutes with aeration. Dilute out the virus in a second series of 4 tubes containing *E. coli* cells in soft agar, as you did before. Plate out the dilutions on agar as above.

Take both these plates home with you, and keep them in a warm spot. By the following morning you should be able to count the blank areas, or plaques, on the plates. On quadrants where many virus particles were plated, plaques will run together ("confluent lysis"). Where no viruses were plated, there will be smooth, confluent growth of bacteria. Count the plaques in those quadrants where they appear clearly. Hold the plates against a black background or up to the light in order to facilitate counting.

From your counts calculate the number of virus particles initially present in the suspension. Calculate also the number of virus particles present at the end of the growth period. How many times greater than the initial count was the final count? How does viral reproduction compare with bacterial reproduction in rate? in its essential mechanism?

Plaque morphology and identification of an unknown phage

Obtain a 4-quadrant nutrient agar plate and label appropriately. Add 1 drop of the *E. coli*

culture to each of 4 tubes of soft agar with a sterile dropper. Add 2 drops of the various phage suspensions to the tubes, mix, and pour the contents onto the appropriate quadrants, as you did earlier.

Take the plates home with you, and the next day study the various plaque shapes, sizes, and appearances. In your notes describe the various plaque morphologies and decide which of the phages matches your unknown.

Filterability of viruses and bacteria

Do the filtration in pairs, but each student should have his own agar plate. Probably several pairs of students will have to take turns using each set of filters. The porcelain filters will be identical, but since four experiments have to be carried out without stopping to sterilize the filters, use only the filter marked "P" for phage and only the one marked "C" for *E. coli*. Also, do not contaminate the porcelain part of the filter by touching it with your finger or placing it on the bench.

Obtain a 4-quadrant nutrient agar plate and label clearly. Two quadrants are for the *E. coli* suspension, before and after filtration, the other two for filtered and unfiltered phage, plus bacteria. Streak a loopful of *E. coli* across one quadrant. (Streaking is a single linear passage of the loop on the surface of the agar.) Place a sterile test tube under the filter marked "C," and apply suction. Pass 20 drops of *E. coli* suspension through the filter. Streak a drop of the filtrate on the appropriate quadrant of your agar plate.

Add 2 drops of T_4 phage suspension to a soft agar tube containing 5 drops of *E. coli*. Pour the tube contents onto the suitable quadrant. Now filter 20 drops of phage and add a drop of the filtrate to a soft agar tube containing 1 drop of *E. coli*. Again plate out the tube contents.

Take this plate home with you and keep it with the others in a warm spot. Examine them the next day, and, if necessary, the day after that. Record your observations.

Further reading

C. A. KNIGHT and D. FRASER, "The Mutation of Viruses," *Sci. Am.* **193**, No. 1, pp. 74–78, July 1955, Reprint No. 59.

S. E. LURIA, "The T_2 Mystery," *Sci. Am.* **192**, No. 4, pp. 92–98, April 1955, Reprint No. 24.

H. FRAENKEL-CONRAT, "Rebuilding a Virus," *Sci. Am.* **194**, No. 6, 42–47, June 1956, Reprint No. 9.

R. Y. STANIER, M. DOUDOROFF, and E. A. ADELBERG, *The Microbial World*, Prentice-Hall, 1957, pp. 365–371.

K. V. THIMANN, *Life of Bacteria*, Macmillan, 1955, pp. 85–94.

EQUIPMENT

Per student

5-ml pipet, sterile
8 dropper pipets, sterile
4 nutrient agar plates
2 wide tubes, sterile
aerator assembly, sterile
bacteriological loop
bunsen burner
14 small test tubes with 1 ml soft agar
5 ml nutrient broth

Per 8 students

suspension of phage T_4 in broth at 10^4 particles/ml (for phage reproduction experiment) (5 ml)

suspension of T_2, T_4, T_{4r}, and unlabeled phage, in broth at 10^2 particles/ml (for phage morphology experiment) (5 ml each)

dropping bottle of *E. coli* culture in logarithm phase of growth (10–20 ml)

2 porcelain filter cylinders assembled in suction flasks, sterile

Per laboratory (30 students)

water bath at 37°C, with aeration assembly

2 water baths at 45°C containing small tubes with soft agar

X PHOTOSYNTHESIS

(Readings: Weisz, pp. 241–263. S.P.T., pp. 95–100. Villee, pp. 94–103. E. I. Rabinowitch, "Photosynthesis," *Sci. Am.* **179**, No. 2, pp. 24–34, Aug. 1948, Reprint No. 34. D. I. Arnon, "The Role of Light in Photosynthesis," *Sci. Am.* **203**, No. 5, 104–118, Nov. 1960, Reprint No. 75.)

The energy that supplies all life on the earth comes ultimately from sunlight, through the process of photosynthesis. Each year plants on the earth reduce about 550 billion tons of carbon dioxide, using about 25 billion tons of hydrogen, and releasing about 400 billion tons of oxygen into the atmosphere. About nine-tenths of this activity goes on in the surface layers of the oceans.

No industrial process yet invented converts light economically into useful forms on a large scale. For this reason our economy still depends largely upon the combustion of fossil fuels, themselves the products of photosynthesis in past ages. We have only recently begun to understand how plants accomplish this feat.

For light to be used, it must be absorbed; and substances which absorb visible light are by that token *pigments*. The pigments which absorb the light used in photosynthesis are found in the *chloroplasts* of green plants, and in similar particles called *chromatophores* in photosynthetic bacteria. The principal pigment of chloroplasts is chlorophyll *a*. Chlorophyll *b* and the yellow carotenoids play secondary roles, transferring the energy they absorb as light to chlorophyll *a* for use in photosynthesis. Photosynthetic bacteria possess a special *bacteriochlorophyll*, and also a number of specific carotenoids.

The net action of light in photosynthesis is to split water, thus providing hydrogen for reductions and eliminating oxygen as a by-product:

$$12H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} 24H + 6O_2.$$

The H atoms supplied in this way are used to reduce CO_2 to carbohydrate and water:

$$6CO_2 + 24H \xrightarrow{18\ ATP} C_6H_{12}O_6 + 6H_2O.$$

Thus the *overall reaction* is

$$6CO_2 + 12H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} C_6H_{12}O_6 + 6H_2O + 6O_2.$$

To fix one molecule of CO_2 in the form of carbohydrate requires not only 4 H atoms but also 3 "high-energy" phosphate bonds of adenosine triphosphate (ATP). The structure of ATP and some of its sources are discussed in Exercise XI. It is now recognized that the

energy absorbed as light by chloroplasts generates not only hydrogen, but also ATP. Indeed, isolated chloroplasts can carry out the whole process of photosynthesis.

Carbohydrate, having been prepared by photosynthesis, is in turn degraded to provide all the cell's energetic needs. The two principal processes for deriving energy by the degradation of sugars are fermentation and respiration. We shall examine both processes in the next laboratory session. Fermentation is the process by which cells derive energy anaerobically, by rearranging the atoms of sugar to yield products of lower energy. Respiration is a combustion, in which sugar is burned with molecular oxygen to yield carbon dioxide, water, and energy in the form of ATP.

Photosynthesis and respiration are opposed reactions. The overall equation of the former is just the reverse that of the latter. Green plants respire in the dark; they simultaneously respire and photosynthesize in the light. The consumption of oxygen is a measure of their respiration; the evolution of oxygen measures their photosynthesis. In the light, with both processes going on simultaneously, the oxygen exchange represents a balance between these opposed reactions. If the light is sufficiently bright, however, photosynthesis may go so much faster than respiration as to dominate the oxygen exchange.

EXPERIMENTS

Analysis of chloroplast pigments

The chlorophylls and carotenoids (xanthophylls and carotenes) are the major pigments of the chloroplasts. These pigments can be extracted from green plant tissues with lipid solvents, and separated by chromatographic adsorption.

Such an extract has been prepared before the laboratory session by homogenizing spinach leaves with 95% ethanol in a Waring blendor. The extract has been filtered, evaporated to dryness, and redissolved in petroleum ether.

With a fine capillary, apply this mixture to the longer side of a 4.5 x 4.5-inch filter paper in a narrow line, 3 inches long, 1 cm from the bottom. Develop the chromatogram with a mixture of 9 petroleum ether : 1 acetone.

In this solvent the carotenes ($C_{40}H_{56}$) move the fastest, followed by the xanthophylls ($C_{40}H_{54}(OH)_2$) and then the chlorophylls *a* and *b*. Outline the visible pigment spots lightly with a pencil. Then examine the paper under ultraviolet light, noting the fluorescence of the various pigments and the presence of any additional spots which were not apparent in visible light. (*Caution:* Recall our earlier warnings not to look into the light.)

Determine where the petroleum ether extract of plant pigments has absorption maxima by looking at this solution through the hand spectroscope. Chlorophyll *a* has a major absorption band at about 680 mμ and chlorophyll *b* at about 665 mμ. Those of you who have time may cut out the two chlorophyll bands on your chromatogram and elute the pigments by leaching out the paper strips in a small test tube with a few ml of acetone. Remove the filter paper with forceps and observe the absorption of these two solutions at the specified wavelengths in the hand spectroscope, or measure it in a spectrophotometer. Which pigment migrated faster on your chromatogram? What is the chemical difference between chlorophylls *a* and *b*? Why is chlorophyll green?

The Hill reaction

The photolytic cleavage of water in the presence of chloroplasts is known as the Hill reaction. It can be represented by the following equation:

$$A + H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} H_2A + \tfrac{1}{2}O_2.$$

In this reaction "A" represents an electron (or hydrogen) acceptor. In plants this is usually the coenzyme TPN. In our experiment we shall use an artificial electron acceptor, the dye 2,6 dichlorophenolindophenol, which is reduced

with the concomitant evolution of oxygen. You will be able to follow the course of the reaction by observing the loss of blue color as the dye is reduced:

$$\text{Dye (blue)} + H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} \text{Dye} - H_2 \text{ (colorless)} + \tfrac{1}{2}O_2.$$

Spinach chloroplasts have been prepared as follows: Leaves were homogenized with 0.5-*M* sucrose solution at 0°C for 30 sec in a Waring blendor. The suspension was then filtered through two layers of cheese cloth. The filtrate was centrifuged at 50 times the force of gravity (50 G) for 10 minutes. The supernatant was then decanted and recentrifuged for 10 minutes at 600 G. The supernatant was decanted and discarded. The pellet at the bottom, containing the chloroplasts, was resuspended in 0.5-*M* sucrose. It is important to keep the chloroplasts at 0°C; they deteriorate rapidly at higher temperatures.

In each of two test tubes, mix:

2 ml of phosphate buffer, 0.1 *M*, pH 6.5;
2 ml of dye solution
(2,6 dichlorophenolindophenol,
2.5×10^{-4} *M*);
0.1 ml of chloroplast suspension (2 drops);
6 ml of distilled water.

Swirl to stir, wrap one tube immediately in aluminum foil to protect it from light, and expose the other to bright light for 10 minutes. Compare. (Protect the chloroplasts from heat radiation by placing a glass tumbler filled with water between the light source and the reaction tubes.)

Devise experiments to show (a) that the chloroplasts and dye must be illuminated *together* to obtain this result; (b) that the reaction depends upon catalysis by enzymes. Include the results of these experiments in your notes.

Oxygen evolution in photosynthesis

Place three leafy sprays of *Elodea* in one test tube of the volumeter described in Exercise VI (pp. 35–36), and fill this tube with 1% sodium bicarbonate solution. Add sufficient solution so that when the rubber stopper is inserted, an air space of about 3 to 6 mm is left between the liquid surface and the stopper. The bicarbonate solution will provide the carbon dioxide used in photosynthesis. Fill the second tube, which as before will serve as thermobarometer, with the same volume of sodium bicarbonate solution.

A 60–100 watt lamp mounted upright in a standard receptacle will be used as light source. Between this lamp and the volumeter place a jar or glass filled with water, to serve as a heat filter, which, by absorbing the infrared (heat) radiation from the lamp, will prevent large temperature changes in the volumeter from distorting the readings. Place the lamp as close to the volumeter as it will go with the heat filter in between. Draw a drop of kerosene into the proximal end of each side-arm, as described in Exercise VI, and close the pinch clamps. Allow the system to equilibrate for 5 to 10 minutes. This equilibration time is needed for the oxygen evolved to saturate the water; thereafter all the gas produced is given off.

Take readings in both side-arms at 2-minute intervals, each time subtracting the reading in the thermobarometer from that in the experimental tube. Go on with the readings until the *rate* of change remains constant through three consecutive readings.

After the rate has stabilized, move the light source to twice the distance from the plant, and after taking readings at this distance double the light distance once again. Assuming that the light intensity is inversely proportional to the distance, plot the rate of oxygen evolution (change of volume in units/min) vs. light intensity (relative). (*Note:* The intensity of light coming from a point source falls off as the square of the distance; i.e., at twice the distance the intensity has fallen to one-quarter. Your lamp, however, is not a point source, particularly if frosted, or equipped with a reflector; and the light intensity declines more nearly in proportion to the distance.)

Optional experiments

(1) In the above experiment the light intensity limits the rate of photosynthesis. If several independent steps are involved in an overall reaction, the rate at which the process goes is determined by the step which has the lowest rate, i.e., the limiting step. Determine at what concentration the bicarbonate becomes limiting at maximum light intensity.

(2) Shake a few pieces of leaf for 3 to 4 minutes in a test tube with petroleum ether. Pour off the petroleum ether into another test tube, replace with methanol, and shake the leaves as before in this solvent, heating meanwhile under the hot-water tap. Pour off the methanol extract into a clean test tube, and compare with the petroleum ether extract. Which contains more pigment? Which is therefore the better solvent for leaf pigments? Now mix both together, shake once vigorously, and let the two layers separate. Which contains more pigment? How do you explain these observations?

(3) Determine in a spectrophotometer the absorption spectra of the mixture of chloroplast pigments and of the separate pigments isolated on your chromatograms. By dividing this job among several students, you may be able to do all of it. To obtain enough of the isolated pigments it will probably be necessary to combine several chromatographed samples in as small a volume of solvent (acetone or ethanol) as the spectrophotometer will handle. The mixture of pigments and the chlorophylls should be measured between 380 and 720 mμ; the carotenoids between 380 and 550 mμ. The instructor will show you how to use the spectrophotometer.

EQUIPMENT

Per student
6 large test tubes
2 small test tubes
quart jar

Per 2 students
volumeter
electric light source
glass tumbler

Per 30 students
95% ethanol
100 ml sucrose (0.5 *M*)
500 ml 2,6-dichlorophenolindophenol (2.5×10^{-4} *M*)
500 ml phosphate buffer (0.1 *M*, pH 6.5)
2 liters of 1% sodium bicarbonate
1 liter absolute methanol
0.5 liter petroleum ether
2 liters acetone
50 filter paper sheets, 4.5″ × 4.5″
fresh spinach leaves
crushed ice
100 fresh *Elodea* stems
fine capillaries for applying pigments to filter paper
roll of aluminum foil

Per laboratory
6 hand spectroscopes
spectrophotometer (the Bausch and Lomb "Spectronic 20" instrument will perform adequately)
cheese cloth, Waring blendor, centrifuge } for instructor before lab
0.5 *M* sucrose
ultraviolet lamp and safety glasses

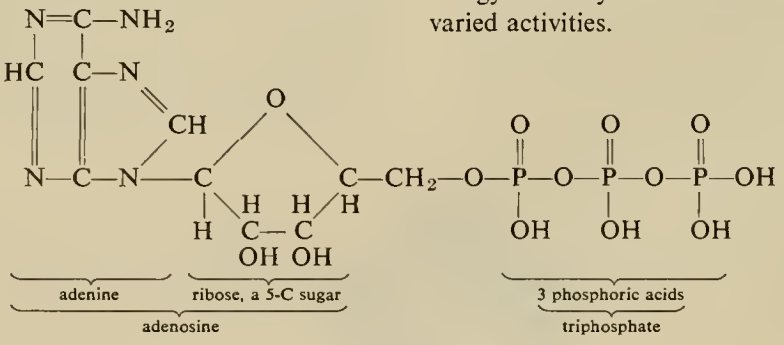
MARINE BIOLOGICAL LABORATORY WOODS HOLE LIBRARY MASS.

XI FERMENTATION AND RESPIRATION

(Readings: Weisz, pp. 319–351. S. P. T., pp. 129–132. A. L. Lehninger, "How Cells Transform Energy," *Sci. Am.* **205**, No. 3, 62–73, Sept. 1961. See also R. Y. Stanier, M. Doudoroff, and E. A. Adelberg, *The Microbial World*, Prentice-Hall, 1957, pp. 147–150, 577–583, and K. V. Thimann, *Life of Bacteria*, Macmillan, 1955, pp. 376–383.)

The great metabolic processes by which cells obtain energy are fermentation and respiration. Fermentation is Pasteur's "life without air"; it provides energy in the absence of oxygen. The essence of this process is the rearrangement of the atoms of a sugar to yield a compound of lower energy, making the difference in energy available to the cell. Respiration is a cold combustion: molecular oxygen is used to burn organic molecules—frequently sugars—to yield carbon dioxide, water, and exactly the same total energy as if the same molecules had been burned in a flame.

In both respiration and fermentation part of the energy is liberated as heat. The organism cannot use this, however, except to warm itself; for living organisms are chemical machines, not heat engines. The energy the cell needs to maintain itself, to make new molecules, grow, move, and reproduce, must be provided in chemical form. Usually this is in the form of adenosine triphosphate, ATP. It takes about 8 kcal of energy per mole to attach the terminal phosphate group to adenosine diphosphate to make ATP (ADP + P $\longrightarrow$ ATP), and this energy is made available again when the terminal phosphate is transferred to other molecules. Such a high-energy phosphate group is frequently designated by the symbol ~P; ATP can be written AP ~ P ~ P. The terminal ~P of ATP is the energy currency with which the cell pays for its varied activities.



The main business of fermentation and respiration is to supply cells with ~P; and the yield of such groups is a measure of the efficiency of these processes.

Yeast, a unicellular organism, can live normally either by fermentation, when no oxygen is available, or by respiration, when oxygen is present:

Yeast fermentation:

$$C_6H_{12}O_6 \longrightarrow \underset{\text{ethylalcohol}}{2C_2H_5OH} + 2CO_2 + 2{\sim}P.$$

Yeast respiration:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + \text{(approx.) } 38{\sim}P.$$

Various other microorganisms ferment sugar to different products, namely butyric acid, acetone, etc., but the principle is always the same. Animal cells also ferment sugar. Muscle cells, for example, are often required to work more rapidly than they can be supplied with oxygen, and do so by fermenting sugar to lactic acid:

Muscle fermentation:

$$C_6H_{12}O_6 \longrightarrow \underset{\text{lactic acid}}{2C_3H_6O_3} + 2{\sim}P.$$

It will be noted that the chemical changes of respiration just reverse those of photosynthesis; similarly the energy of sunlight stored in sugars by photosynthesis is released in respiration to make high-energy phosphate bonds. Green plants carry out both processes, photosynthesis in the light, and respiration at all times.

You have already measured photosynthesis in *Elodea* by the rate of oxygen evolution in the light. Now we will measure respiration in a higher plant by the rate of oxygen consumption. As you see by the above equation for respiration of sugars, one molecule of CO_2 is produced for each molecule of O_2 consumed, so that, according to Avogadro's law, one would expect no change in gas volume. We shall absorb CO_2 as fast as it is formed, however, with soda lime (a mixture of solid sodium hydroxide and calcium hydroxide). *Write the equation for this process.*

The rate of respiration varies greatly over the life span of many organisms, being most rapid during growth and development and slowing down with maturity. Pea seedlings that are 3 to 4 days old have very rapid rates of respiration, and thus were chosen for this experiment. Since the products of respiration are also the reactants of photosynthesis, it is advisable to hold the latter process to a minimum during your measurements. For that reason, the pea seedlings were germinated in the dark, and so lack chlorophyll. They green rapidly, however, when exposed to light, so keep them shaded.

EXPERIMENTS

Respiration

We shall be working again with the volumeter described originally in Exercise VI (pp. 35–36). Fill one of the test tubes to within 2 inches of the top with pea seedlings, tapping the test tube against your hand to pack the seedlings. Insert a cotton plug over the seedlings, and layer about 1 inch of soda lime over the cotton plug, to absorb all carbon dioxide. Be sure that no soda lime touches the seedlings. The second test tube, which again will act as thermobarometer, should be filled to 2 to 3 inches from the top with water, to approximate the volume occupied by solid material in the experimental tube.

Insert the rubber stoppers and adjust indicator drops in the side-arms, this time placing the drop in the experimental tube near the *distal* end of the scale. Clamp the escape tubes and wait about 5 minutes for equilibration. Take readings in both side-arms at 3-minute intervals, each time subtracting the reading in the thermobarometer from that in the experimental tube, until the *rate* of change in the experimental tube becomes constant. This measures the rate of oxygen consumption.

If you now knew the rate of oxygen consumption minus carbon dioxide production, you could calculate the rate of evolution of carbon dioxide. Figure out how to do this yourself; then *do* the experiment.

The rate of carbon dioxide production divided by the rate of oxygen consumption is the so-called respiratory quotient (R. Q.). What do you find it to be? What does the equation for the respiration of sugar, shown above, predict? What would the R. Q. be if an organic acid (e.g., palmitic acid, $C_{15}H_{31}COOH$) or an amino acid were being respired rather than a sugar? Can you conclude from your R. Q. what types of metabolites are being respired by these pea seedlings?

Fermentation

Working in pairs, stir about $\frac{1}{16}$ of a yeast cake into 20 drops (1 ml) of glucose solution in one of the volumeter test tubes. The other tube, to serve as thermobarometer, should contain 3 ml of water. Adjust kerosene drops at the proximal ends of the side-arms in the volumeter, and take readings in both side-arms every minute, each time subtracting the volume changes in the thermobarometer from those in the experimental tube. It may take a few minutes for a constant rate of change to be established, since the sugar solution must first be saturated with carbon dioxide.

Repeat this experiment, this time stirring the yeast into 1 ml of galactose solution, to test the ability of yeast to ferment this sugar.

To verify that the gas produced in fermentation is carbon dioxide, make use of the fact that carbon dioxide reacts with calcium hydroxide to yield an insoluble precipitate of calcium carbonate. Pour the yeast suspension in glucose solution from the volumeter test tube into a small test tube, connect a gas-delivery tube, and let the gas bubble through limewater (calcium hydroxide solution; this is prepared by stirring powdered calcium hydroxide in water for a few minutes, and filtering). If the gas production is not rapid enough, add more yeast and glucose to your fermentation mixture. It may also take a little time for the carbon dioxide produced to break through the foam.

Yeast ferments glucose, fructose, and mannose indiscriminately. Why? Does it ferment galactose? Why? It will help you to construct and compare the molecular models of these substances.

The total energy change in fermenting a mole of glucose (how many grams?) to alcohol and carbon dioxide is about 20 kcal. If this change makes 2 moles of ~P available, what is its efficiency in producing *useful* energy?

Calculate the *weights* of carbon dioxide and ethyl alcohol produced in fermenting one mole of glucose. How do they compare?

The total energy change in respiring one mole of glucose is 672 kcal. If this produces 38 ~P, what is its efficiency?

EQUIPMENT

Per student

2 wide test tubes
microscope
slide and cover slip

Per 8 students

4 test tubes, with assembly consisting of No. 0 rubber stopper with 5-mm diameter hole; 2″ piece of 6-mm diameter glass tubing
4 volumeters
4 gas-delivery tubes: 6″ piece of rubber tubing $\frac{3}{16}$″ in diameter and a 6″-long dropper tube
2 dropping bottles of 10% glucose
2 dropping bottles of 10% galactose
2 250-ml beakers
funnel
filter paper
cotton (nonabsorbent)
soda lime
calcium hydroxide
cake of yeast
pea seedlings

XII, XIII

THE ARRAY OF LIVING ORGANISMS

(Readings: Weisz, Chapter 29. S.P.T., Chapter 19. Villee, pp. 83–84. R. Y. Stanier, M. Doudoroff, and E. A. Adelberg, *The Microbial World*, Prentice-Hall, 1957, Chapters 3–6. Other readings are listed at appropriate places within the exercise.)

So far this semester we have studied general properties and processes in living organisms, emphasizing components and reactions rather than the specific organisms in which they were examined; and so we shall go on doing. Yet we have already encountered a fair variety of organisms, and next semester we shall deal with many more. Also next term we shall study more highly integrated phenomena as they appear in more complex organisms.

Up to now, whenever we have encountered a new organism, something has been said of its biological position. The time has come to go beyond such a piecemeal approach and to gain a view of the entire array of living things.

Biological order as history and experiment

At first glance nature appears to abound with an enormous diversity of living organisms. Careful examination of the different forms, however, enables us to group them on the basis of similarities in anatomical organization, embryological development, chemical constitution, and other criteria. The catalogue of types that results is highly useful in itself, both in reducing the diversity to manageable proportions and in enabling us to ascertain readily the general nature of an organism once we are aware of its name or of enough of its properties to place it in the classification scheme.

But emerging from this classification, and indeed woven inseparably into its fabric, we find two tremendous concepts: that of origin and descent; and that of progressive adaptation, of ceaseless problem-solving, accompanying the ceaseless expansion of organisms into every environment that can support life, working out in all environments their universal problems of nutrition and reproduction. That is, we come out not only with a history, but one that involves direction, indeed many simultaneous directions; in essence, the history of the exploration of this planet as an abode for life. It is this that transforms what might otherwise be a tedious catalogue into a profound intellectual adventure.

The dominant view that guides the construction of a scheme of classification—once one has simply made order, grouping similar organisms together and separating the groups in proportion to their differences—is that all living organisms, plant and animal alike, are linked together by descent from common ancestors, from which they evolved along separate paths to their present state of divergence. Although each group

TABLE 1

Plants	Animals
Perform photosynthesis	Live on organic materials, ultimately supplied by plants
Possess functional chlorophyll	Chlorophyll is not present, except rarely as functionless pigment, retained from plant diet
Use starch as the principal food reserve	Use glycogen or fat as principal food reserve
Have rigid cell walls	No cell walls
Display no active movement (sessile)	Usually display active movements
Grow indefinitely to various sizes and shapes	Usually grow to fixed size and shape

now alive has as long a history of evolution as any other—no contemporary organism is the ancestor of any other contemporary organism—certain groups have changed relatively little over long periods of time. From these, and from genuine ancestors preserved as fossils, it is possible to construct a genealogy of living things, a tree of life, that shows the lines of ancestry and divergence among living forms. On such a tree, all present-day organisms have equivalent status, at the tips of branches. It is the stems and branch points that express their evolution.

A first approach: the three kingdoms

All living things may be divided into three great kingdoms: plants, animals, and protists:

PLANTS	ANIMALS
Seed plants	Vertebrates
Ferns	Invertebrates
Mosses, liverworts	Sponges, jellyfish

PROTISTS

Plant-like			*Animal-like*
Blue-green algae	Algae	Slime molds	Protozoa
Bacteria	Fungi		

The protists are mainly single-celled organisms, sometimes containing many nuclei within a single cell membrane (multinucleate). Some are multicellular, but then display little or no differentiation of tissues to perform specific functions. We regard such aggregated protists as colonial, to distinguish them from the multitissued organisms. Usually the cells of such colonial forms can also live independently, and can give rise by division to new colonies. Most protists are small; but red or brown seaweeds may achieve great size and very complicated shapes—giant kelps may be 150 feet long—yet with little differentiation of tissues. (Various authors include different groups within the protists. Simpson, *et al.*, include the protozoa among them, but place the algae among the plants. Weisz does not use the category protist at all, and puts the animal-like protists among the animals and the plant-like protists among the plants. Stanier, *et al.*, in *The Microbial World* group them as here, and provide an excellent discussion of the relations among them.)

Fortunately most of you have good, working notions of animals and plants, and could probably decide fairly accurately into which group to place even unfamiliar forms. The characteristics that divide these two kingdoms are summarized in Table 1.

Orders of classification

The members of each kingdom are arranged in a hierarchy of groupings. The major groups

TABLE 2

	Paramecium	Corn	Lobster	Man
Phylum (Subphylum)	Ciliophora	Tracheophyta Pteropsida	Arthropoda	Chordata Vertebrata
Class	Ciliata	Angiospermae	Crustacea	Mammalia
Order	Holotricha	Monocotyledonae	Eucarida	Primates
Family	Hymenostomata	Gramineae	Decapoda	Hominidae
Genus	*Paramecium*	*Zea*	*Homarus*	*Homo*
Species	*caudatum*	*mays*	*americanus*	*sapiens*

are called *phyla;* from these one works down through smaller and smaller divisions, finally to the double name, genus and species, by which any single type of organism is called.

Some idea of the task involved in classifying and naming living organisms may be gained from the realization that there are about 300,000 living plant and over a million animal species. It is a little like assigning a meaningful status and relationships to everyone in Los Angeles.

The main taxonomic divisions can best be illustrated by classifying a few familiar organisms from the three kingdoms, as we have done in Table 2. How much of this kind of thing do we want you to know? We want you to know the really important things that are involved in and lie behind such classification schemes. We will try to point them out to you as we go along, and they are summarized in the diagrams which follow.

A depressing thing about much of the technical terminology used in classification is that it keeps changing. Even at any given time, we find great disagreement involving even the main categories. For example, the terms "Tracheophyta" and "Pteropsida" used in the classification of corn in Table 2 are characterized as "abandoned" by a recent authority (H. C. Bold, *The Plant Kingdom*, Prentice-Hall, 1960). Indeed, what are eight plant phyla in Simpson, *et al.*, have now been reclassified by Bold into 24 "divisions."

What saves this situation from its zealots are two things: common names, which do stay in use; and the possibility of expressing most of the fundamental relationships in plain English. Thus in place of the above technical classification of corn, already obsolete according to some authorities, we can describe it safely as a vascular, flowering, seed plant, one of the grasses—indeed, Indian corn or maize.

Another point important for us is that we classify only to the extent that serves our needs. For example, most biologists would say of *Paramecium* that it is a protozoan and a ciliate, and let it go at that. As for the lobster, most of us are content to know that among the Arthropods it is a decapod crustacean. For the most part, the remaining terms used in Table 2 would be used only by specialists.

So relax, use common names and ordinary English as much as you like, but do learn to recognize the important groups of protists, animals, and plants, and learn as much as you can of the relationships among them. Read your text, and use the following pages as a guide to what we most want you to learn.

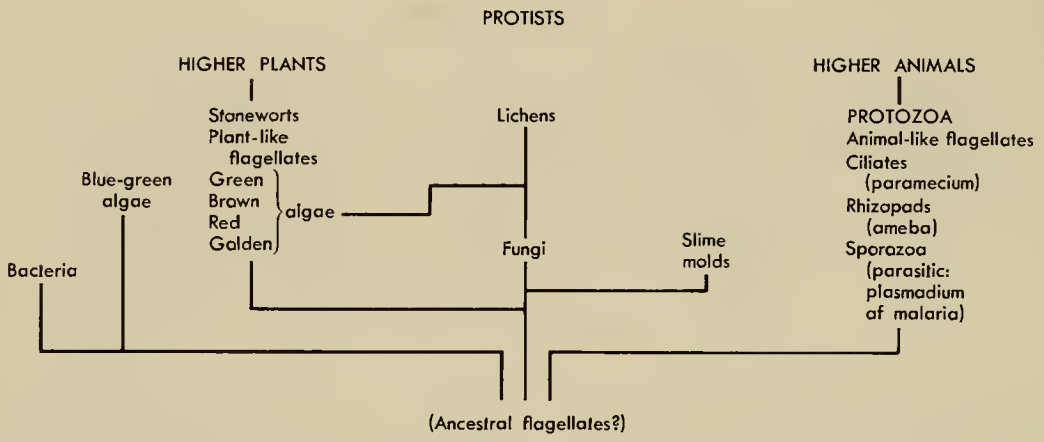
THE PROTISTS

Guidelines

(1) Close relations between bacteria and blue-green algae as structurally simplest protists.

(2) The "flagellate line."

(3) Colonial algae as first approaches to differentiated multicellular organisms.



Each of these groups is classified at present as a distinct phylum, except that the term "Protozoa" now designates a subkingdom within either the protist or animal kingdom; and the flagellates as a whole constitute the Phylum Mastigophora (whip-bearers), which includes the plant- and animal-like flagellates and the dinoflagellates.

Among the protists the bacteria and blue-green algae stand somewhat apart. They are highly successful groups, particularly the bacteria; yet structurally they represent the simplest of living cells, and so are sometimes spoken of as "lower" protists, though not ordinarily to imply an ancestral position. The blue-greens, like the true algae, are photosynthetic, evolving oxygen in this process. The bacteria may be photosynthetic or not, but never evolve oxygen in photosynthesis. These two groups share the following further properties:

(1) Small size, including the smallest living cells.

(2) No separate nucleus, surrounded by a membrane.

(3) No chloroplasts.

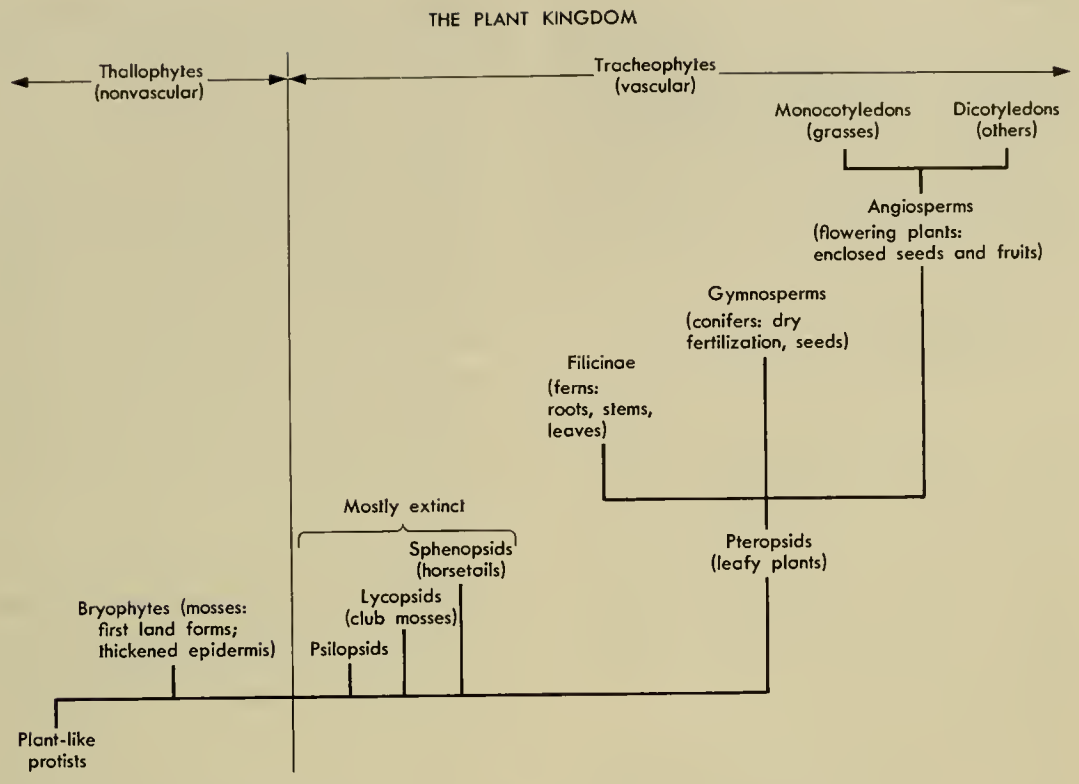
(4) Motionless cytoplasm.

(5) A chemical distinction, the exclusive possession of diaminopimelic acid.*

The flagellates may provide the thread that binds the protists together, and leads off in the directions of the multitissued plants and animals. It is this that has prompted the thought of a flagellate ancestor, probably photosynthetic, which may have given rise on the one hand, with some structural retrogression, to the bacteria and blue-greens, and on the other to the more highly developed protists, both plant-like and animal-like. It is as though, starting with an ancestral green flagellate, the photosynthetic capacity had been exploited in developing the algae, and the motility exploited in developing such protozoa as the ciliates.

Modern flagellates include both green, photosynthetic types (e.g., *Euglena* and *Chlamydomonas*, both of which you have studied), and colorless animal-like forms, such as the trypanosomes responsible for African sleeping sickness. Flagellated cells form one stage in the development of slime molds. We have also the colonial green flagellates, some of which display a first differentiation of function, certain cells being specialized for reproduction (recall *Volvox*). These may represent first approaches to the formation of the multitissued plants. On the other hand the sponges, the first of the animal phyla, possess characteristic flagellated collar cells that greatly resemble free-living flagellated protozoa. The cells of sponges also display an

*$HOOC—CHNH_2—(CH_2)_3—CHNH_2—COOH$, a carboxylated lysine.



extraordinary independence on occasion. A sponge can be pressed through cheese cloth so that all the cells are separated. Left to themselves, they reaggregate to form a new sponge.

Stoneworts, though a small group, are the most complex algae. You already know one of them, *Nitella*.

Lichens are composite associations of algae with fungi, living together to their mutual benefit (symbiosis), the alga photosynthesizing and the fungus providing water and a source of nitrogen for both partners.

The slime molds display both plant-like and animal-like characteristics, passing through stages of free-living, unicellular flagellates and amebae. Then the ameboid individuals migrate together and form a great multinucleate slug, bounded by a single membrane. This may then differentiate into a beautiful fruiting body, carrying a bulb containing spores at the end of a long stalk, very plant-like in appearance.

THE PLANT KINGDOM

(Readings: S. P. T., Chapter 21. Weisz, Chapter 30. Villee, Chapters 11 and 12. Optional readings are C. P. Swanson, *The Cell*, Prentice-Hall, 1960, and H. C. Bold, *The Plant Kingdom*, Prentice-Hall, 1960.)

Principal groups and numbers of species

Flowering plants (250,000)

Conifers (600)

Gingko (maidenhair tree) (1)

Cycads (100)

Ferns (9500)

Horsetails (25)

Club mosses (1000)

Mosses (14,000)

Liverworts (9000)

Total: approximately 300,000 species

Guidelines

(1) Alternation of haploid gametophyte and diploid sporophyte generations.
(2) Progression from gametophyte-dominance (mosses, liverworts) to sporophyte-dominance.
(3) Emergence from water to land: vascularization.
(4) Mosses, liverworts, and ferns as the amphibia among plants.
(5) Development of dry fertilization in conifers and flowering plants.

A major difference between plants and animals, one which runs throughout the entire plant kingdom and extends back among the colonial algae, involves their reproductive habits. Animals are almost invariably diploid (the nuclei of their cells contain *pairs* of chromosomes, forming a double set) except for the mature germ cells (gametes: eggs and sperm), which are haploid (i.e., each contains a single set of chromosomes).

In contrast with this, plants alternate a diploid, spore-bearing (sporophyte) generation with a haploid, gamete-bearing (gametophyte) generation. It is true that in higher plants the gametophytes, male and female, are very small, and live upon the diploid sporophyte, which forms the main body of the plant. Yet in lower plants these relations are reversed, and all plants display the basic pattern of alternation of generations. This is how it works:

(1) Diploid sporophyte by meiosis (cell division with reduction from double to single chromosome number) yields haploid spores.
(2) Haploid spore by ordinary cell division (mitosis) yields a haploid gametophyte, bearing haploid gametes.
(3) Fertilization of an egg by a sperm restores the diploid number of chromosomes, and by mitosis yields a new diploid sporophyte.

The forerunners of modern plants were undoubtedly aquatic. They had to face neither the problem of conveying food and water over relatively long distances, as must be done in the larger land plants, nor providing devices by which the germ cells could find one another, which is no problem in water.

Mosses and liverworts, most of which made the transition from water to land, have compromised with both these problems. They are nonvascular (i.e., lack conducting vessels), and hence are restricted to a small size, which keeps them close to the ground. Also, at the time of sexual fertilization they must manage to collect enough water for the sperms to swim to the eggs. In these plants the gametophyte is the dominant, free-living generation; it is what we mainly see as the plant. The sporophyte is a relatively small structure that remains permanently attached to the gametophyte.

With the evolution of specialized tissues to overcome the difficulties of terrestrial living, plants came to cover almost all the land masses. The primary step in this development was the evolution of vessels to conduct water and dissolved materials throughout the organism. This step is so important that the plant kingdom has traditionally been divided into two subkingdoms: the nonvascular thallophytes (algae, fungi, bryophytes) and the vascular tracheophytes, the higher land plants. The tracheophytes have also developed further specialized tissues, leaves, roots and stems, which have aided in the colonization of the land. The simplest tracheophytes, the psilopsids, club mosses, and horsetails, most of which are now extinct, display the beginnings of all these developments.

In all the tracheophytes the sporophyte is the dominant generation; it is what we see as the plant. In ferns, the gametophyte is still free-living, though reduced to a very small size. In the more advanced groups the gametophytes are represented by only a few cells.

The leafy plants (Pteropsids) have diverged in the course of their evolution to form three large groups: the ferns, conifers, and flowering plants. All of them have well-differentiated roots, stems, and leaves. The ferns, however, have still not won freedom from one condition of aquatic life: they still need water in which the sperms,

which develop in one part of the tiny gametophyte, can swim to the eggs, which develop in another part of the gametophyte, at the bottom of a cleft.

The conifers have made further steps in adaptation to land life. They have developed two types of spores, which give rise respectively to male and female gametophytes. The male gametophyte, now a pollen grain, is dispersed by the wind or by insects, so eliminating the need for water. The female gametophyte is entirely parasitic, living always within the tissues of the sporophyte. On fertilization of the egg by the sperm delivered by a pollen grain, it yields a sporophyte embryo, which, provided with food and a protective coat, is the *seed*. On being planted, this develops into the mature sporophyte.

In the flowering plants (angiosperms) the reproductive systems achieve further refinement. Stems and leaves are modified to form flowers, which contain the gametophyte generation. (We shall study flower structures in detail next semester, so we need not go into them deeply now.) The gaily colored flowers with their perfumes and nectars attract insects and birds, which willy-nilly transport pollen from one flower to another, ensuring efficient fertilization. Fertilization of the egg within the ovary of a flower leads, as in conifers, to the growth of an embryo sporophyte, which, with its surrounding tissues and protective coat, constitutes the seed. The angiosperms, however, go one step further than the conifers, enclosing the seed in a fruit, which develops from tissues of the flower. The fruits may be eaten by animals, which disseminate the seeds over the countryside.

The angiosperms are the most complex and successful land plants. They include about $\frac{3}{4}$ of all living plant species. They divide into the so-called monocots and dicots on a rather trivial basis, whether the cotyledons, the food-containing, leaflike structures within the seeds, are single (as in a corn seed) or double (as in a peanut). The monocots include the grasses, and several minor groups, palms, lilies, onions, and orchids. The dicots are almost everything else.

THE ANIMAL KINGDOM

(Readings: S.P.T., Chapters 22 and 23. Villee, pp. 195–207; Chapters 14 and 15. Weisz, pp. 667–702. An excellent additional source that does almost the whole job in a couple of hours of pleasant reading is the Golden Science Guide, *Zoology*, by H. S. Zim, H. I. Fisher, and R. W. Burnett. Also, see an excellent discussion in Weisz, Chapters 29 and 31, and the fine pictures in Ralph Buchsbaum's *Animals Without Backbones*, University of Chicago Press, rev. ed., 1948.)

Principal groups and numbers of species

PHYLUM CHORDATA (50,000)
- Subphylum: vertebrates
  - Classes: mammals
    - birds
    - reptiles
    - amphibia
    - bony fishes
    - cartilaginous fishes
    - placoderms (extinct, armored, jawed fishes)
    - jawless fishes (Cyclostomes)
- Subphyla (3) of protochordates (Amphioxus, acorn worms, tunicates)

PHYLUM ECHINODERMATA, "spiny-skinned" (6000)

PHYLUM ARTHROPODA, "jointed-legs" (1,000,000)
- Classes: insects
  - arachnids (spiders, horseshoe crab)
  - crustacea (crabs, lobsters, barnacles)

PHYLUM MOLLUSCA (100,000)
- Classes: gastropods (snails, slugs, whelks)
  - pelecypods (clams, mussels)
  - cephalopods (squid, octopus, nautilus)

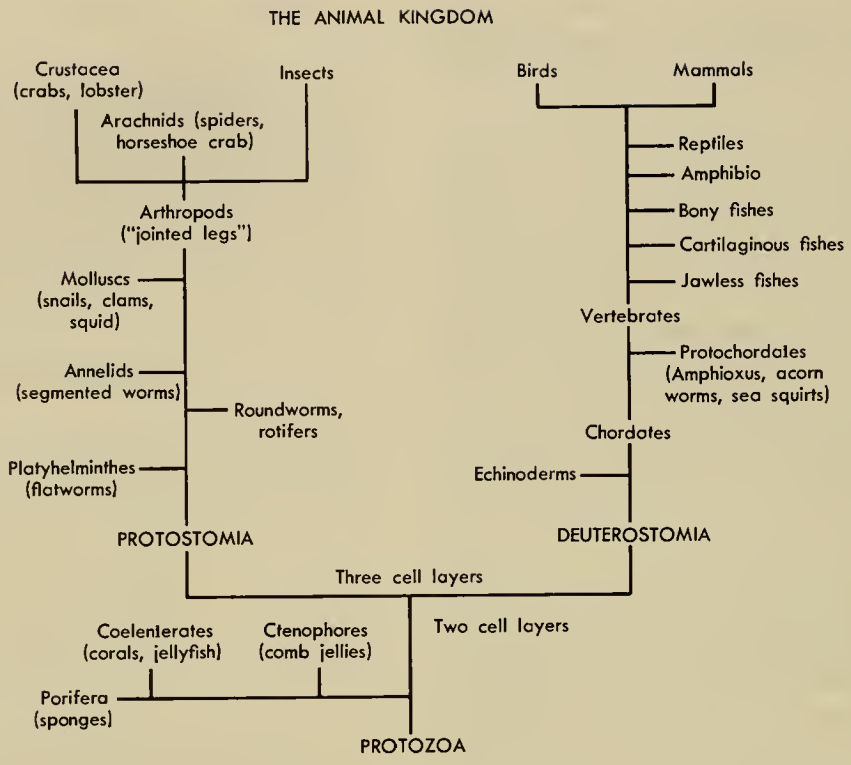
PHYLUM ANNELIDA, segmented worms (10,000)

PHYLUM NEMATODA, roundworms (10,000)

PHYLUM PLATYHELMINTHES, flatworms (10,000)

PHYLUM COELENTERATA, corals, jellyfishes, hydrozoa (10,000)

PHYLUM PORIFERA, sponges (15,000)



Of the approximately 1,200,000 species of living animals, about 97% are invertebrates and about 75% are insects.

Guidelines

(1) Two cell layers (sponges, coelenterates) to three cell layers.

(2) Development of a true body cavity (coelom) lined with mesoderm.

(3) Adaptations for emergence from water to land and air.

(4) Grouping of annelids, arthropods, and molluscs as protostomes ("annelid superphylum") and of echinoderms and chordates as deuterostomes ("echinoderm superphylum").

The coelenterates and comb jellies are built of two layers of cells, the internal endoderm lining the digestive cavity, and the external ectoderm. All further phyla add a third layer between these two, the mesoderm.

The coelenterates and flatworms have only a single opening into the digestive cavity, which therefore serves as both mouth and anus. The higher phyla possess tubular digestive systems, open at both ends. In arriving at this condition, the annelids, arthropods, and molluscs (protostomes) convert the primitive opening to a mouth, and break through a new opening for the anus. In the echinoderms and chordates (deuterostomes), the primitive single opening becomes the anus, and a new opening is broken through to form the mouth.

One of the most important developments is the formation of a body cavity (coelom) lined with mesoderm, in which the internal organs lie. The sponges and coelenterates have no mesoderm, and no such cavity. The flatworms have a solid mesoderm, and no cavity. The round worms have a restricted mesoderm, and apart

from it an internal space lined by ectoderm and endoderm. The higher phyla all possess a true coelom.

In the protostomes, the mesoderm is formed by cells which wander in from the ectoderm and bud off the endoderm to form a solid layer, in which the cavity later develops that becomes the coelom. In the deuterostomes, the mesoderm is formed by outpocketings of the endoderm to form hollow pouches, the cavity of which is the coelom.

WORK ASSIGNMENT

These two laboratory sessions will be devoted to a study of the diversity of organisms, animal and plant. Representative plants and animals of all the major groups will be on display, and the student should study each of them carefully. There will also be a group of unlabeled plants and animals to examine and to compare with the labeled specimens.

Working independently, place the unlabeled organisms within their major categories: phyla for the most part; but also subphyla among the vascular plants, and classes among the Pteropsids; similarly, classes among two of the animal phyla, the Arthropods and Chordates. In a few sentences and perhaps a sketch, defend each of your identifications. Hand in your results at the end of the period.

To prepare for these laboratory sessions, and to supplement them, students should, if possible, spend two or three hours in the nearest accessible museum that has a display of representative organisms of the major plant and animal phyla. For such an excursion to be meaningful, the student should first abstract the pertinent material from our list of readings, and probably have with him the charts that appear in this manual, as well as the outline that follows. Notes should be taken about the phyla observed, their distinctive characteristics, the range of organisms they include, and the relationships among them.

A Short Guide to Plant and Animal Classification

During the year a variety of organisms will be used in the laboratory. It will be worth while to attempt to classify them. As an aid in doing this, an abbreviated guide is included here. Various authors disagree on minor points of classification, but practically any textbook of botany or zoology can be consulted for more details.

PLANT KINGDOM*

Plants are usually considered as organisms with stiff cell walls and with chlorophyll.

* Adapted from C. A. Villee, Jr., *Biology*, 4th ed., Saunders, 1962.

Subkingdom Thallophyta (Gr. *thallos*, young shoot; *phyton*, plant). Plants not forming embryos. These are the simplest plants without true roots, stems, or leaves; there is little differentiation of tissues.

1. PHYLUM CYANOPHYTA (or Myxophyta) (Gr. *myxa*, mucus; *phyton*, plant). The blue-green algae. Chloroplasts and nuclei not distinct.

2. PHYLUM EUGLENOPHYTA (Gr. *eu*, well, true; *glene*, pupil of the eye or socket of a joint). The Euglenoids.

3. PHYLUM CHLOROPHYTA (Gr. *chloros*, green; *phyton*, plant). The green algae. Contain distinct nuclei and chloroplasts. *Spirogyra* and *Oedogonium*.

4. PHYLUM CHRYSOPHYTA (Gr. *chrysos*, gold; *phyton*, plant). The yellow-green algae, the golden-brown algae, and the diatoms.

5. PHYLUM PYRROPHYTA (Gr. *pyrrho*, red; *phyton*, plant). The Cryptomonads and dinoflagellates.

6. PHYLUM PHAEOPHYTA (Gr. *phaios*, dun-colored; *phyton*, plant). The brown algae; multicellular, often large bodies; large seaweeds such as *Fucus*.

7. PHYLUM RHODOPHYTA (Gr. *rhodon*, rose; *phyton*, plant). The red algae; multicellular; usually marine; sometimes impregnated with calcium carbonate.

8. PHYLUM SCHIZOMYCOPHYTA (Gr. *schizein*, to cleave; *mykes*, fungus; *phyton*, plant). The bacteria.

9. PHYLUM MYXOMYCOPHYTA (Gr. *myxa*, mucus; *mykes*, fungus; *phyton*, plant). The slime molds. Made up of protoplasm containing many nuclei but without division into distinct cells. Movement is ameboid.

10. PHYLUM EUMYCOPHYTA (Gr. *eu*, well, true; *mykes*, fungus; *phyton*, plant). The true fungi. This phylum contains the Phycomycetes (bread and leaf molds), the Ascomycetes (yeasts, mildews, cheese molds), the Basidiomycetes (mushrooms, toadstools, and rusts), and the Fungi Imperfecti (fungi which are difficult to classify, such as that causing athlete's foot).

Subkingdom Embryophyta (Gr. *embryon*, to swell; *phyton*, plant). Plants forming embryos.

11. PHYLUM BRYOPHYTA (Gr. *bryon*, moss; *phyton*, plant). No conducting tissue; multicellular; terrestrial; alternation of sexual and asexual generations (the prominent plant is the sexual generation, the gametophyte). Mosses, liverworts, and hornworts.

12. PHYLUM TRACHEOPHYTA (Gr. *tracheia*, artery: *phyton*, plant). Vascular plants.

Subphylum Psilopsida: rootless and leafless vascular plants.

Subphylum Lycopsida: clubmosses; small green leaves and a simple conducting system.

Subphylum Sphenopsida: horsetails; jointed stems and scalelike leaves.

Subphylum Pteropsida: complex conducting systems and large, conspicuous leaves. This subphylum is divided into three major classes: Filicinae (ferns), Gymnospermae (conifers, cycads, most evergreens and shrubs—no true flowers or ovules present—the seeds are born naked on the surface of the cone scales), and Angiospermae (flowering plants with seeds enclosed in an ovary). The Angiosperms may be subdivided into the Dicotyledons and Monocotyledons. The dicots have embryos with two cotyledons (seed leaves); most flowering plants belong to this subclass. The grasses, lilies, and orchids, however, are monocots, their embryos having only one seed leaf.

ANIMAL KINGDOM*

Ten questions are particularly useful in distinguishing phyla of the animal kingdom:

(1) Unicellular or multicellular?
(2) Diploblastic or triploblastic? (Is the body composed of two layers, the ectoderm and endoderm, or are there three layers, ectoderm, endoderm, and mesoderm?)
(3) Body saclike or built on tube-within-a-tube plan?
(4) True digestive cavity present or absent?
(5) Segmented or nonsegmented?
(6) Asymmetry, bilateral symmetry, or radial symmetry?

* Adapted from M. F. Guyer, *Animal Biology*, 3rd ed., Harper Brothers, 1941.

(7) Appendages present or absent? If present, jointed or not?
(8) What is the nature and position of the skeleton (exoskeleton or endoskeleton)?
(9) Notochord or vertebral chord present or absent?
(10) What is the structure and position of various organ systems?

I. **Protozoa** (unicellular animals)

1. PHYLUM PROTOZOA (Gr. *protos*, first; *zoon*, animal). Single cells or loosely aggregated colonies of single cells. *Amoeba*, *Euglena*, *Paramecium*, *Stentor*, *Volvox*, etc.

II. **Metazoa** (multicellular animals containing specialized tissues)

A. ***Parazoa*** (no true digestive cavity)

2. PHYLUM PORIFERA (L. *pours*, pore; *ferre*, to bear). Sponges. Sessile; aquatic; diploblastic; radially symmetrical body consisting of cylinder closed at one end: budding and folding of body often present; digestion does not occur in central cavity but rather in individual cells; generally a skeleton present.

B. ***Enterozoa*** (true digestive cavity present)

(a) *Enterocoela* (only cavity in the body is the digestive cavity)

3. PHYLUM COELENTERATA (Gr. *koilos*, hollow; *enteron*, intestine). Hydroids, jellyfishes, sea anemones, and corals. Diploblastic; body may be tubular (polyp) or bell- or umbrella-shaped (medusa); in some organisms these forms alternate during life cycle; budding to form colonies is common; body is a double-walled sac; body cavity is not separate from digestive tract; radiate symmetry.

4. PHYLUM CTENOPHORA (Gr. *ktenos*, comb; *phoros*, bearing). Sea walnuts, comb jellies. Triploblastic (ectoderm, endoderm, and mesoderm); radial combined with bilateral symmetry; body with eight meridionally arranged rows of swimming plates; a few species are ribbon-shaped.

(b) *Coelomocoela* (coelom present, tube-within-tube structure)

(i) Nonsegmented

5. PHYLUM PLATYHELMINTHES (Gr. *platys*, broad; *helminthos*, worm). Flatworms. Bilaterally symmetrical; no true segmentation, flattened dorsoventrally, no blood vascular system; no anus; mostly parasitic. The flatworms are triploblastic but don't have a definite coelom. They do, however, have gonocoels which represent a primitive form of coelom.

6. PHYLUM NEMATHELMINTHES (Gr. *nematos*, thread; *helminthos*, worm). Roundworms. Bilaterally symmetrical; unsegmented; usually long and thin; most often contain an alimentary tract with mouth and anus; body cavity present; papillae or spines at anterior extremity of body; both parasitic and free-living forms.

7. PHYLUM ROTIFERA (L. *rota*, wheel; *ferre*, to bear). Common small aquatic forms; usually found in fresh water but also may be marine and parasitic; ciliary movements on anterior end suggest a rotating wheel; nervous system present; body enclosed in flexible cuticle; body usually roughly cylindrical tapering at posterior end to form a foot; well-developed digestive system with mouth, pharynx, glandular stomach, intestine, and anus.

8. PHYLUM BRYOZOA (Gr. *bruon*, moss; *zoon*, animal). Moss animals and sea mats. Small; aquatic, sessile, unsegmented; usually colonial; ciliated tentacles surround mouth; U-shaped intestine with anus near mouth; colonies often look superficially like hydroid colonies.

9. PHYLUM BRACHIOPODA (L. *brachium*, arm; Gr. *podos*, foot). Lamp shells. Unsegmented body covered with calcareous bivalve shell; mouth is between two spiral, ciliated arms which lie within shell; many fossil forms.

10. PHYLUM ECHINODERMATA (Gr. *echinos*, hedgehog; *derma*, skin). Sea lilies, starfish, sea cucumbers, sea urchins. Marine; radi-

ate symmetry in adults; most forms with spiny skin; triploblastic with large coelom and distinct alimentary canal; calcareous plates provide protective exoskeleton; tube feet for locomotion.

11. PHYLUM MOLLUSCA (L. *molluscus*, soft). Chitons, snails, slugs, whelks, clams, scallops, oysters, ship worms, squids, octopuses. Unsegmented; appendages not jointed; usually a shell and mantle; "foot" usually present.

(ii) Segmented

12. PHYLUM ANNELIDA (L. *annelus*, a little ring). Segmented worms such as earthworms, marine worms, leeches. Blood vessels, excretory organs, and nervous system segmentally arranged; distinct coelom; ventral double nerve cord of "ladder type"; appendages not jointed.

13. PHYLUM ARTHROPODA (Gr. *arthron*, joint; *podos*, foot). Crustaceans such as crayfish, water fleas, crabs, barnacles; centipedes, millipedes; insects; spiders, mites, ticks, scorpions. Segmented (but body cavity is continuous and without transverse septa; segmentation shown internally in arrangement of organs); groups of segments tend to fuse into larger regions (head, thorax and abdomen); paired, jointed appendages; exoskeleton; nervous system of "ladder type"; main longitudinal blood vessel dorsal to alimentary canal.

14. PHYLUM CHORDATA (Gr. *chorde*, cord). Internal skeleton with a notochord sometime during life history; gill clefts in throat sometime during life history; central nervous system is tubular and dorsal. The phylum Chordata consists of several subphyla, the most important of which is the Craniata (or Vertebrata), the backboned animals. Among the members of the subphylum are the Cyclostomata (lampreys and hagfishes), the Elasmobranchii (sharks and rays), Pisces (bony fish), Amphibia (salamanders, frogs and toads), Reptilia (lizards, snakes, turtles, crocodiles), Aves (birds), and Mammalia (mammals).

EQUIPMENT

Laboratory exhibit of plants and animals representing all major groups, labeled and unlabeled

XIV VERTEBRATE ANATOMY

(Readings: Weisz, pp. 201–223; also browse through Chapters 12, 13, 14, 20 and 21, looking especially at the diagrams and photographs. S. P. T., pp. 117–158, and browse through to p. 220. Villee, pp. 234–242, and browse through Chapters 17–25.)

Our first semester in the laboratory was devoted primarily to the forms and activities of individual cells. We stressed mainly widespread or universal similarities among cells, whether animal, plant, or protist.

This semester we will study the aggregations of cells which compose the tissues and organs of the higher organisms. A *tissue* is a group of cells which display common functional and/or morphological properties. Usually in a tissue the cells are bound together to form sheets, layers, or more-or-less solid structures; but this need not always be so. The circulating blood cells in vertebrates, for example, constitute a tissue. The higher animals are composed mainly of four types of tissue: epithelial, connective, muscular, and nervous. Higher plants also possess four primary types of tissue (protective, meristematic, "fundamental," and conductive or vascular). An *organ* is a structure composed of two or more tissues that performs a specialized function.

Just as we started last semester by surveying types of cells, the first two laboratory sessions this semester will be devoted to studying a few typical higher organisms. In this first period we shall study vertebrate anatomy; next week we will examine the anatomy of higher plants.

The work this week may be done in pairs, one partner dissecting a rat, the other a frog. As you dissect, compare the similarities and differences between these vertebrates. Learn the names, locations, appearance, and functions of the various organs you encounter. Your textbook will help, as also will wall charts posted in the laboratory. You and your partner should try to dissect in the same body area at the same time.

A systematic procedure, outlined below, will direct you to the major structures. Strike out on your own, if you like; but if you do, plan ahead how you are going to go about it.

When you have finished examining an organ, it is often helpful to remove it. If you choose to do this as you go along, slice into each structure you remove. Note its internal appearance, whether it is solid or possesses a cavity, and whether it appears homogeneous or differentiated into distinct regions. Keep your animal and the organs you have removed from it moist, and they will retain their natural shape and appearance throughout the period.

Stained sections of the major organs will probably be available from your instructor. Although this will be a good time to look at them,

we will examine such sections in greater detail later, so don't spend too much time with them now.

The animals will be given to you alive but anesthetized. One of the problems that biologists face, and that you are facing now, is how to examine what goes on inside animals without causing them pain. "Anesthesia" literally means lack of feeling or sensation. We try to achieve this either by rendering the higher centers of the brain functionless with the use of a narcotic ("anesthetic") or by destroying those centers. Whatever one does in this regard should be done skillfully and quickly. If you pith your own frog, know exactly what you are going to do, and be ready to do it before you make the first move. All the biologists we know take a lot of trouble with such procedures. Speed and skill are of the essence. If an animal needs to be killed in the course of a laboratory procedure, one takes similar precautions, trying whenever possible to kill in one stroke an animal that has been handled gently up to that point.

The particular point of the procedures we have used here with the rat and frog is to abolish pain, yet permit you to examine the organs in a functional state. Take full advantage of this opportunity, and be prepared to begin as soon as the animal is ready. Work as fast as you can, consistent with care and thoroughness.

The rats will be given an overdose of barbiturate by the instructor at the start of the period. Watch the anesthesia take hold. This in itself is instructive. The barbiturates inhibit the higher centers of the brain first—the cortical centers— lessening the animal's coordination. The first effect is a staggering gait. (This is what ethyl alcohol does to us.) Gradually the animal becomes immobile, though its reflexes still respond to external stimuli. The reflexes are controlled through lower centers of the brain or through the spinal cord, both of which are more resistant to narcosis. Eventually the reflexes also cease to respond, yet the animal continues to breathe. The respiratory center in the brain stem is extremely resistant to narcosis. Why do you think this is so?

At this point, when the animal no longer responds to stimuli, but is still breathing, begin the dissection.

The frogs are "anesthetized" by destroying (pithing) their brains. One of the instructors will demonstrate this procedure, and will pith your own frog if you wish.

Pithing is done by quickly inserting a dissecting needle, directed forward into the skull, at the point where it joins the backbone, meanwhile moving the point of the needle from side to side as far as it will go, to cut as many as possible of the nervous connections. If you hold a frog gently but securely, and bend its head a little downward, you can find a little depression at the back of the head that marks its joint with the backbone. This is where the needle should be inserted, with one swift motion that gets inside the brain cavity, stirring as it advances.

THE RAT

First look carefully at the external appearance of your animal, and compare it with the frog. Note differences in texture of the skin. Where does the head end? What do you see in the way of ears? How many digits are on the feet? Feel such major body landmarks as the rib cage, backbone, and the connection of the backbone with the skull. Where is the heartbeat strongest? Note the breathing movements.

Lay the animal on its back, and slit open the skin from the jaw to the genital openings. Be careful not to cut through the underlying tissues. Separate the skin laterally, using your fingers or the blunt end of the scalpel. (If you have difficulty here, the instructor will demonstrate.)

Note: The popular image of a biologist has him dissecting with a scalpel. The truth is that the cutting edge of the scalpel is used relatively little. Most cutting is done with scissors, and most dissecting is done by prying, pushing, and lifting things apart rather than cutting. You will probably find the spade-shaped, blunt end of the scalpel more generally useful than the blade. When you really want to slice, as, for example,

an organ that you have removed, a single-edged razor blade ordinarily does better than the scalpel.

Open the abdomen about midway down from the rib cage. Extend a longitudinal slit forward to the ribs (be careful not to go beyond) and back to the genital openings. Make lateral slits down the side of the body wall parallel to the rib cage. Observe the packing of the abdominal contents; poke about, noting connections and relative positions. Don't be afraid to lift the organs away from one another. Watch the slow peristaltic movements of the intestine. Pull the liver down and observe the lung through the transparent part of the diaphragm.

Cut through one side of the diaphragm and the rib cage on that side, staying close to the midline. Note the collapse of the lung. (Why does it collapse?) Pull open the rib cage and observe the heartbeat. Describe the motions. Do all the parts beat simultaneously? Collapse the lung on the other side and remove the ribs and sternum, over the heart. This will allow you to inspect the heart more closely. Try to trace the major vessels to and from the heart (the *vena cava* and *aorta*). Can you distinguish arteries and veins? It might help to remove the white *thymus* gland, which is found anterior to the heart and may obscure its atria (auricles). (You may keep your animal's heart beating longer and observe the expansion of the lungs by inserting a dropper pipet attached to a rubber tube into the trachea and breathing for the rat. The instructor will demonstrate this for you.)

By this time, anoxia is probably overcoming the heart and other tissues. The muscles may twitch, but these are "automatic" responses; the animals feels nothing. After you have observed the heart, cut a slit into the end of the ventricle with your scalpel to bleed the animal. Take it over to the sink, and wash away the blood. Now start dissecting the abdominal contents.

First, examine the *liver*. The veins draining the intestines pass through the liver on their way to the heart. (Of what importance do you think this might be in the light of liver function?) Trace these veins as best you can to and from the liver. Remove the liver carefully, freeing it as far as possible from its attachments to surrounding tissues. Examine the excised liver, noting its consistency, lobes, etc. (Make such a cursory examination of every organ you remove.)

The *stomach* is found just below the liver on the animal's left. Find the *esophagus*, following it to where it penetrates the diaphragm. (We will dissect it completely later.) Examine the *intestinal tract*. Pull it out, noting the mesentery that attaches the intestine to the body wall (all the organs in the body cavity are surrounded and supported by mesenteries). Note the fanlike arrangement of blood vessels in the mesentery. Note the large *caecum* of the rat. (Do we have a caecum?) Below the caecum, the digestive tract is called the *colon*; above, the intestine. Next find the *pancreas*, buried in the mesenteries just below the stomach. Starting at either end, free and remove the intestinal tract in one long string. How long is the intestine? Comparing your height and the length of the rat, how long would you estimate your own intestinal tract to be?

Look next at the *spleen*, located below and to the right of the stomach. Why is the speen so dark in color? What is known of its function?

Study the *kidneys*. Try to trace the *ureters* to the *bladder*. Find the *adrenal glands* in the fat just above the kidney. These are round, brownish bodies, easy to miss. Note the prominent blood vessels leading from the kidney to enter or leave the heart through the *vena cava* and the *aorta*. Remove the kidneys and adrenal glands. (Be sure to slice open these organs and look at their cross sections.)

Now move to the *thorax*. If a model of the human thorax is available in your laboratory, study it before going on with your dissection. Look at the lungs, heart, esophagus, and major vessels that run forward from the heart and then back along the ridge of the spinal column. Follow the aorta and vena cava backward as far as you can. Try to reach the place where they fork before entering the legs. Remove the heart; identify the ventricles and atria. Slice it to look at the internal structure. (We will study the mammalian heart in some detail later in the

course, so don't linger with it here.) Next find the *trachea*; look for the *thyroid* glands clinging to its side about two-thirds of the way to the mouth. Free the trachea from the esophagus and carefully dissect the *bronchi* and lungs free.

Next look at the internal *reproductive organs.* If your rat is a male, push the *testis* up into the abdomen. Note the tubular *epididymis* attached to the testis. Follow the *ductus deferens* to the seminal vesicles. Slit open the epididymis and squeeze some of its contents onto a microscope slide. Add a drop of saline and cover with a cover slip. Observe the sperm under the microscope. If your preparation is reasonably fresh, the sperm should still be motile. Describe their motions. In the female, find the *ovaries.* Follow the *fallopian tubes* to the *uterus.*

Now turn to the *muscular system.* First skin the animal. This can be done easily with blunt dissection and the fingers. The pelt can be removed in one piece. Dissect one foreleg and hind leg. Identify as many muscles as you can. (The best procedure here is to peel off the overlying connective tissue, cut across the muscle or its *tendon* at one end, and strip the whole muscle back.) Note the large nerves entering the limbs; these are seen best by excising the muscles on the under side of the forelimbs or on the rear of the hind limbs.

Next cut through one side of the jaw. Look at the tongue and remove it. Find the opening of the esophagus. How do the teeth differ from ours? How many different types of teeth do you see?

Rongeurs will be available for exposing the brain. First remove the eyes, and then cut across the skull between the eyes. Making small snips, cut away the brain case overlying the brain. (Be careful; the brain is very fragile!) When you have exposed the brain, lift it from the anterior end. Note the cranial nerves (especially the optic nerves) on the underside. Free the brain, and either remove it entirely or continue the dissection down the spinal cord. Note the nerves coming from the spinal column. Note also the way the cord bulges where the nerves to the fore limbs come off. Where does the spinal cord terminate?

If you continue to dissect down the spinal cord, it may be helpful to turn the animal on its back. Follow an arm or leg nerve out into a limb. Eventually remove both cord and brain. Cut the brain through longitudinally. Note the grey and white matter (especially in the cerebellum). What do they represent? Cut across the cerebral hemispheres, and note the tracts of white matter. Cut other cross sections, and attempt to follow some tracts.

THE FROG

Follow the directions given for the rat as far as they are applicable. You should be able to find almost all the organs mentioned in the rat dissection. On opening the abdomen, however, note immediately that the frog has no diaphragm. How does it breathe?

Look at the beating heart under the dissecting microscope. Follow the major vessels. Can you see the blood flowing? Look at all the body contents of the frog under the dissecting microscope. When you find interesting things, show them to your partner.

Follow the dissection guide given for the rat. (If you have a female frog, the abdominal contents may be filled with two masses of dark spherical bodies. These are the ovaries filled with growing eggs. Remove them immediately on beginning the abdominal dissection.)

You will have an easier time dissecting the visceral organs than your partner. You may have trouble identifying the kidneys. They are long, narrow organs found close to the midline on the dorsal body wall. The adrenal glands are the long yellow structures applied to the kidney's surface. The testes are small white bodies suspended from the kidney. Compare your dissection with that of your partner and note the differences in the two animals.

The frog nerves and muscles will remain excitable for most of the day. Stimulators will be available (your instructor will demonstrate them for you). Try stimulating various nerves and muscles; learn their names, and show them to your partner.

You will not be able, of course, to dissect the brain, but the frog *eye* is large and can be examined. Identify its parts. Excise the eye, and carefully cut around its equator. Lift off the cornea, iris, and lens (which will come away together). Then with a thin blunt instrument, lift the retina and pigment layers from the back of the eye cup.

EQUIPMENT

Per student
dissecting tools
microscope (dissecting and compound)
slides and cover slips
dropper and 12″ rubber tubing
dissecting pan
frog or rat

Per 8 students
stimulator and electrodes
box of pins
25 cc saline (0.9%)

Per 30 students
4 rongeurs
bottle of nembutal (50 mg/ml) (50 cc)
1-cc syringe and No. 27 needle
5 pithing needles
1 pr. gloves for handling rats
stained sections of major frog and rat organs
wall charts of frog and rat dissections
skeletons of frogs and rats
model of human thorax

XV ORGANIZATION OF HIGHER PLANTS; THE TRANSPORT OF SAP

(Readings: Weisz, pp. 171–199; 231–239. S. P. T., pp. 55–63; 137–141; 368–378. Villee, pp. 104–106; 117–129. Review the discussion of the plant kingdom in Exercises XII and XIII. V. Grant, "The Fertilization of Flowers," *Sci. Am.* **184**, No. 6, 52–56, June 1951, Reprint No. 12. M. H. Zimmerman, "The Movement of Organic Substances in Trees," *Science*, **133**, Jan. 13, 1961, pp. 73–79.)

This week we shall examine the organization of flowering plants (angiosperms), which represent the peak of plant evolution, just as the vertebrates studied last week represent the peak of animal evolution. We shall also inquire into an important aspect of their function, the transport of sap, which plays a role in vascular plants comparable with the circulation of blood in animals; and into the osmotic relations of plant cells, upon which the transport of sap largely depends.

Surely you are already familiar with the gross division of higher plants into stems, roots, leaves, and flowers. In the course of this period we shall examine these organs more closely, dissect a flower and a fruit, and examine under the microscope the tissues of which such organs are composed.

We shall begin, however, by setting up experiments on the rise of sap, and on plasmolysis. Once these have been started, they need only occasional attention; and while they are going on, you can examine the anatomy of plants and plant structures.

WATER MOVEMENT IN PLANTS

One of the major problems in the life of vascular plants is the transport of sap. This flows in two streams, one generally downward, carrying organic molecules prepared by photosynthesis in the leaves; the other upward, carrying water and dissolved ions absorbed from the soil by the roots. For the plant to survive and grow, both streams must penetrate to all its tissues.

Of these two streams, the upward stream of water and salts from the roots is the larger and more continuous. A fraction of it supplies the downward stream, and to this degree we may speak of the flow of sap as a "circulation." A further fraction contributes to the growth of the plant, and is retained in new tissues. Much of the ascending water, however, is lost by evaporation from the leaves.

The upward stream presents the major problem. To bring sap from the roots to the top of a high tree demands a very large force. The highest trees—California redwood, for example, and

eucalyptus—may be over 300 feet high. How sap is raised to such heights is a problem that has plagued plant physiologists for generations. A prevalent type of theory, entertained for a time, was that as water evaporated from the leaves, it left a vacuum in the ascending vessels (xylem), which drew water upward. Even if one could establish a perfect vacuum in the upper vessels of a tree—a very unlikely possibility—this would provide a pressure of only 1 atmosphere to raise the sap. One atmosphere pressure raises water about 34 feet. To bring sap to the top of a 300-foot tree would require about 9 times this force, that is, about 9 atmospheres pressure.

OSMOTIC PRESSURE AND PLASMOLYSIS

There is little doubt that the major force for the ascent of sap in plants is osmotic pressure. Whenever two solutions are separated by a semipermeable membrane—a membrane that readily passes water and small molecules, but blocks the passage of larger molecules and some ions—water tends to flow through the membrane from the more dilute to the more concentrated solution. It is easy enough to understand why. The more concentrated side in terms of dissolved molecules is the more dilute side in terms of water. Suppose that there were pure water on one side of such a semipermeable membrane, and a 10% solution of molecules that could not go through the membrane on the other side. At every instant large numbers of molecules collide with the membrane from both sides. On the side containing pure water, of every 100 molecules that hit the membrane, 100 would go through. On the other side, of every 100 molecules that hit the membrane, only 90 would go through, that is, only the molecules of water. The result is a tide of water into the solution, exercising a water pressure (the osmotic pressure) that tends to raise its level to such a point that the added weight of water pressing downward counterbalances the further entrance of water. The height to which the level of the solution rises on the more concentrated side is a measure of its osmotic pressure.

A simple formula makes this relationship quantitative. You know from last semester that 1 mole of any gas in a volume of 22.4 liters has a pressure at 0°C of 1 atmosphere. In exactly the same way, 1 mole of solute that cannot get through a semipermeable membrane, distributed in a volume of 22.4 liters of water, has an osmotic pressure at 0°C of 1 atmosphere. That is, 1 mole of such nondiffusing material dissolved in 22.4 liters of solution (a 0.045 *M* solution) exerts an osmotic pressure that can raise water 34 feet. To raise water 300 feet by this means would require only about a 0.4 *M* solution.

It is important to note that what one is concerned with in accounting for osmotic pressure is the total concentration of particles that do not penetrate the membrane, whatever their nature. The "particles" may be all alike, or greatly mixed, small or macromolecules, or even molecular aggregates, indeed, anything dispersed in water that does not go through the membrane. The essential factor is the degree to which such particles dilute the water on both sides of the membrane.

The protoplasm of plant cells contains dissolved substances which do not readily diffuse through the semipermeable plasma membrane. Hence when a cell is immersed in water, more water molecules diffuse into the cell than diffuse out. The net tide of water into the cell inflates it, producing a pressure against the cell wall. This *turgor pressure* keeps the cell plump and relatively rigid. Conversely, drying the cell or placing it in a more concentrated solution, by withdrawing water, decreases the turgor pressure, causing the cell to soften or *wilt*.

When a plant cell is placed in water, water enters until the turgor pressure is large enough to counteract its further (net) entrance. At this point the turgor pressure, driving water out of the cell, equals the osmotic pressure, drawing water in. In this state of equilibrium, water has not stopped moving in and out of the cell, but it is moving in and out at equal rates.

THE ASCENT OF SAP

The cells in the root have a higher osmotic pressure than the salt solution in the soil. This so-called *root pressure* draws water in from the soil and pushes it upward through the vessels of the xylem. A second source of osmotic pressure originates in the leaves. Here water is continuously evaporated from mesophyll cells, and water vapor finds its way to the exterior through the small openings in the leaves called stomata. This loss of water from the leaves (transpiration), since it concentrates continuously the contents of their cells, creates an osmotic pressure that tends to draw water into them from adjoining cells, and eventually from the sap-filled conducting vessels of the xylem.

As already noted, if this removal of water from the vessels of the xylem acted by creating a vacuum at the top of the sap column, that could at most develop a pressure of 1 atmosphere and could raise sap at most 34 feet. It is now realized, however, that owing to the great *cohesion* of water (why has it so great a cohesion?), a continuous column of water can support a tension of at least 20 to 30 atmospheres and perhaps much more before breaking. Added to this, the cellulose walls of the xylem and its conducting vessels not only imbibe much water (up to 30 to 40% of the plant's dry weight) but also bind the water of the sap columns by powerful electrostatic forces and hydrogen bonding to the —OH groups of cellulose. Very long columns of sap can be lifted by this combination of forces: transpiration of water pulling from the top, the internal cohesion of the sap column, soaking up of water through imbibition by the cell walls, and the adhesion of the sap columns to the walls of the vessels.

This entire view of the process is spoken of as the transpiration-cohesion-tension theory of sap rise. It appears principally to account for the rise of sap in trees in full leaf, with root pressure as a secondary force. Transpiration pulls and root pressure pushes the sap upward. In the early spring, of course, before the leaves appear, there is little if any transpiration, and the sap must ascend mainly by root pressure.

EXPERIMENTS

Plasmolysis

Sucrose, though a small molecule, enters cells only very slowly. If a plant cell is placed in a sucrose solution whose concentration is greater than that of the total dissolved contents of the cell (i.e., a hypertonic solution), water leaves the cell. When enough water has left, the protoplasm of the cell within its plasma membrane contracts away from the cell wall. This process is called plasmolysis. The concentration of sucrose at which plasmolysis just becomes detectable is equivalent to the osmotic concentration of the cell contents.

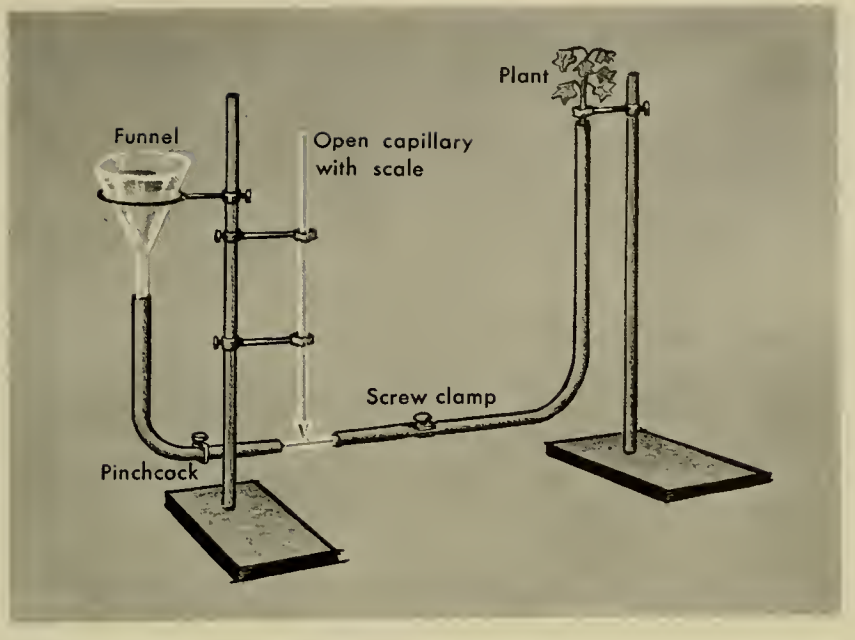
We shall determine in this way the osmotic concentration of epidermal cells of the red onion. With scalpel and tweezers remove strips of epidermis. The color of these cells is due to a red, water-soluble anthocyanin pigment, and will help you to detect the first withdrawal of the cytoplasm from the cell wall.

Place strips of epidermis in a graded series of sucrose solutions ranging in concentration from 0.1 to 0.6 *M*. Leave them for 30 to 60 minutes, and then determine the degree of plasmolysis by observing them under low power in the compound microscope. From your observations estimate the approximate osmotic concentration of the cells. What osmotic pressure (in atmospheres) should they develop when placed in water? How high could this osmotic pressure raise a column of water?

Transpiration

Water absorbed by the roots travels through the vessels of the xylem, which form a continuous conducting system from the young roots to the mesophyll tissue of the leaves. Most of the water absorbed by a plant in leaf is lost by evaporation from the surfaces of the mesophyll cells. The water vapor finds its way through intercellular spaces in the mesophyll to the external air via the stomatal openings.

A geranium plant is available for each two students. The device for measuring transpira-



tion (called a potometer) should be set up as shown in the diagram. Keep the stem of the cut plant in water until the rest of the setup is ready to receive it. Wire all joints for a tight fit.

Before attaching the plant, be sure all other joints are tight, and fill the entire apparatus with water. Shut off the funnel, which serves as a water reservoir, from the rest of the apparatus with the pinchcock.

When the potometer is ready, slice off the bottom one-fourth to one-half inch of the stem *while it is still under water;* then quickly push it into the open end of the rubber tubing which is filled with water. Be sure no bubble of air is caught in the joint. Use the tubing stretchers to aid in inserting the plant; then coat the outside of the joint with a little vaseline. (Be sure not to get vaseline on the cut surface!) Clamp the plant so that the cut end is at about the same level as the top of the capillary. Add water if needed to the funnel, so that its level is higher than the top of the capillary. Then open the pinchcock so as to fill the capillary with water, and tightly close it again.

As water is lost from the leaves by transpiration, the water level in the capillary drops. Let this go on for a convenient interval, and note the time and change of water level. By reopening the pinchcock, bring the water level in the capillary back to near the starting point, and repeat the measurement.

After a few consecutive readings are in good agreement with one another, calculate the rate of transpiration, per hour, day, and year. Direct a light on the geranium plant; does the rate of transpiration increase? What is the effect of a stream of air blowing on the leaves?

PLANT STRUCTURE

We have already noted that the tissues of higher plants, like those of higher animals, can be divided into four types. *Meristematic* tissue is responsible for the production of new cells, and the growth of the plant. The new cells formed from the meristems may differentiate into any one of the other three types of plant tissue. The *protective* tissues (epidermis and cork) comprise the outermost layers of the plant. The "fundamental" tissues are more variable in function and type. Some of them provide support (fiber tissue: *sclerenchyma* and *collenchyma*); others are concerned with photosynthesis (*parenchyma*). There are two kinds of *conductive*

tissues, the *xylem* which transports water and dissolved minerals upward from the roots, and *phloem* which transports food materials from the leaves to all parts of the plant. Phloem cells usually do not have as thick walls as xylem, and may be distinguished from them on this basis.

Stem anatomy

Begin by looking at the slides of the herbaceous (green) angiosperm stems, alfalfa (*Medicago*), a dicot, and corn (*Zea mays*), a monocot. Both contain easily recognized vascular bundles of both xylem and phloem. In dicots, however, the vascular bundles are arranged in a ring, while in monocots the vascular bundles are scattered throughout the stem. This is one of the major differences between mono- and dicots.

A second significant difference is that dicots may retain some meristematic tissue in the stem (called *cambium*) which lies between the xylem and phloem, and which may lay down new xylem and phloem. Most monocot stems, on the other hand, lack a cambium.

Identify the following cell types in the stems: epidermis, parenchyma, phloem, xylem, fiber tissue, and cambium.

Next study the cross sections of the 2- to 4-year-old woody stem of the tulip tree (*Liriodendron*). Here the cambium has produced new (secondary) xylem and phloem. The earlier (primary) xylem has been left behind as orderly rows of cells (wood), but the primary phloem has been crushed to a thin layer which lies just beneath the epidermis. The xylem cells that form in the spring of the year are bigger than those that form in the summer and fall. The latter also have thicker walls. These differences account for the annual rings visible in a tree trunk.

You will find also some tangential and radial sections of stems of *Liriodendron* and *Thuja* (arborvitae).

There will also be pieces of various woods available, cut in different planes. Examine these, correlating their grains with the microscopic sections.

Leaf anatomy

Examine the fixed and stained cross section of a leaf of privet (*Ligustrum*). Note the following layers:

1. Cuticle and epidermis of the upper surface of the leaf.

2. Mesophyll. This is made up of two layers, the palisade parenchyma and the spongy parenchyma. (Of what advantage are the intercellular spaces in the spongy parenchyma?) The veins (vascular bundles) are distributed through the mesophyll. The xylem here again has thicker walls than the phloem and is stained pink.

3. Epidermis and cuticle of the under surface of the leaf. Note the specialized epidermal cells called *guard cells*. They occur in pairs with a pore or *stoma* (plural: *stomata*) between them. Gas exchange and water loss occur through the stomata. The guard cells are capable of swelling or shrinking, through changes in osmotic pressure, depending on the light and other conditions. When they are turgid, the stomata are open; when they wilt, the stomata close. This is an important regulatory mechanism in the leaf.

Root anatomy

Study the prepared slides of median longitudinal sections of root tips of corn (*Zea mays*). Identify the root cap, the meristematic zone, the zone of cell elongation, and the root hairs. The meristematic zone provides all the new cells for the growth of the root.

Also examine the slides of a mature root of the dicot, buttercup (*Ranunculus*). Three layers should be distinguished: the outer *epidermis*, from which the root hairs arise; the *cortex*, made up primarily of cortical parenchymal cells, which may contain starch grains (stained violet); and the *stele*, a cylindrical area enclosing the conducting elements. Note that the xylem (thickened walls, stained red), in the center, is in the form of a star. The phloem cells are located between the arms of the xylem.

Flower and fruit

Each pair of students will be provided with a flower (probably a tulip). Study the following structures, progressing from the outside inward. The *pericanth* consists of an outer series of green leaves (*sepals*), not always present, and colored, modified leaves (*petals*). The inner sex organs are called *stamens* (male) and *pistils* (female). A "perfect" flower has both stamens and pistils. Each stamen consists of a slender stalk at the end of which is the *anther*, which produces the *pollen* grains. Examine some pollen under the microscope. Recall that this represents the male gametophyte.

The pistil consists of an enlarged basal portion, the *ovary*, which supports a slender tube, the *style*. The tip of the style is slightly flattened, forming the *stigma*. When the flower is receptive to fertilization, the stigma becomes sticky, helping it to retain the pollen grains. Inside the ovary are several ovules, which produce the female gametophytes. (Read carefully the discussion of the formation of the egg and pollen, and fertilization in Weisz, pp. 556–559; S. P. T., pp. 368-378; Villee, pp. 182–184.)

After fertilization and formation of the embryo, the ovule tissues harden, forming a *seed coat*. In some plants, the seeds are retained in the ovary, which develops into a fruit. Examine either an apple or pear, cut in cross sections. Identify as many structures as you can. After that, it's yours!

EQUIPMENT

Per student

compound microscope
porcelain spot plate
slides and cover slips
The following prepared slides:
stem of *Medicage* (alfalfa)
stem of *Zea mays* (corn)
2- to 4-year old woody stem of *Liriodendron* (tulip tree)
tangential section of *Liriodendron*
radial section of *Thuja* (arborvitae)
leaf of *Liqustrum* (privet)
root tip of *Zea mays*
root of *Ranuiculus* (buttercup)

Per 2 students

medium-size funnel
capillary tubing (about 3 ft) or 0.1 ml calibrated pipet
T-tube
punch cock and screw clamp
2 ring stands with 4 clamps
rubber tubing
400-ml beaker
geranium plant
tulip or other flower
apple or pear

Per 8 students

graded sucrose solutions (0.1 to 0.5 *M*) (20 ml)
vaseline
flexible wire for joints
red onion

Per laboratory

assorted pieces of polished wood
wall charts of stem, leaf, and root anatomy

XVI BLOOD AND CIRCULATION

(Readings: Weisz, pp. 433–457. S.P.T., pp. 141–153; 163–168. Villee, Chapters 16 and 17. Also B. W. Zweifach, "The Microcirculation of the Blood," *Sci. Am.* **200**, No. 1, 54–60, Jan. 1959, Reprint No. 64. C. J. Wiggers, "The Heart," *Sci. Am.* **196**, No. 5, May 1957, Reprint No. 62. W. B. Wood, Jr., "White Blood Cells vs. Bacteria," *Sci. Am.* **184**, No. 2, 48–52, Feb. 1951, Reprint No. 51. M. B. Zucker, "Blood Platelets," *Sci. Am.* **204**, No. 2, 58–64, Feb. 1961.

One of the principal problems facing a cell as part of a multicellular organism is that it no longer has free access to the external environment. To obtain water, salts, and organic nutrients, to get rid of wastes, and for gas exchange, it must depend on some sort of circulatory system. The importance of the circulation in maintaining an animal can hardly be overestimated. By far the largest cause of death in man is failure of the circulation.

Beyond its nutritive and excretory roles, the circulatory system in vertebrates performs an essential function in defending the animal from invasions of foreign organisms and foreign molecules. A failure of these defense mechanisms can lead to death as surely as the failure in the nutritive and excretory functions of the blood.

The blood of higher animals is a complex tissue. It may be separated by centrifugation into a fraction composed of *cells*, and a cell-free liquid fraction called the *plasma*. The plasma is a complex solution of proteins, sugars, salts, and other substances. One of the plasma proteins, *fibrinogen*, is the precursor of the insoluble *fibrin* of the blood clot. The remainder of the plasma after the clot has been removed is called *serum*. For both the nutritive and defensive roles of the circulatory system, both cells and plasma are needed.

Let us first consider the *nutritive* function of the blood. Many substances are carried in water solution in the plasma and are transported to the cells in this fashion. Other substances are adsorbed on proteins in the blood and are carried in this way. Gas exchange presents further problems. A little oxygen and somewhat more carbon dioxide can be dissolved in the plasma; but the major transport of both these gases in vertebrates depends upon the red pigment, hemoglobin, an iron-porphyrin-protein. The hemoglobin is carried in specialized cells, the red blood cells or *erythrocytes*. About as much hemoglobin is packed into these cells as they can possibly hold. Some 30% of the red blood cell or 95% of its dry weight is hemoglobin. The red blood cells are nonmotile, and do little more than carry hemoglobin. In mammals these cells lose their nuclei before maturing; and as you would expect, from that point on they run down

metabolically, dying after an average life of about 120 days.

Human red blood cells are about 7.5 microns in diameter and have a biconcave disc shape which facilitates gas exchange. They are present in great numbers in the blood; a normal young man may have nearly six million erythrocytes per cubic millimeter of blood. (If the human blood volume is 6 liters, how many new red blood cells must be produced per day to keep the total number constant?)

For *defense*, the body depends on both plasma proteins and cells. The plasma contains a special group of proteins, called *antibodies*, which combine with and hence inactivate foreign proteins, viruses, or polysaccharides, and also cause invading bacteria to clump together. Each antibody is specific for the substance or type of cell with which it reacts. Somehow our defense machinery knows the shapes of our own proteins and leaves them alone. When foreign proteins or polysaccharides called *antigens* are introduced into the circulation, antibodies against them are quickly synthesized.

The cells of the defense system, the white blood cells or *leucocytes*, in marked contrast to the red blood cells, are motile and highly active. They can travel about in the blood stream, or by going through the wall of a blood vessel can wander out into the tissues and tissue spaces. They move more or less as does an ameba, by flowing in one direction or another. When infection strikes, they quickly travel to the invasion site in great numbers. There they destroy large numbers of invading organisms by ingesting them, a process called *phagocytosis*, and also release special substances which help organize the defense. The pus formed in and around an infection consists of dead white blood cells.

A specialized group of white blood cells, the plasma cells (*plasmocytes*), produce antibodies. White blood cells can be divided into two groups: the round, smooth-nucleated *lymphocytes* and the *granulocytes*, which have irregularly lobed nuclei. White blood cells are slightly larger than red blood cells, and are present in considerably smaller numbers (about 8000 per cubic millimeter of blood). During infection, however, their number increases enormously, and this increase provides a sensitive warning that an infection is present.

A third group of elements in the blood, the *platelets* (thrombocytes), is involved in clotting. When a blood vessel is cut open, an interlacing network of fibrin forms a clot which eventually closes the wound. This process is complicated, involving the platelets, calcium ions, and the plasma proteins thrombin and fibrinogen (thrombin is a proteinase which activates fibrinogen by hydrolyzing off part of it, turning it into fibrin).

In addition to its nutritive and defensive activities, the blood provides a constant internal environment for the cells and tissues of the body. In a mammal the pH, temperature, and sugar concentration of the blood are held within very narrow limits. This relative stability of the internal environment makes it possible for a mammal to experience enormous changes in the external environment without damage. The great nineteenth century physiologist, Claude Bernard, was thinking of this when he said, "The constancy of the internal environment is the condition of a free life."

During this period you will prepare and examine a stained smear of your own blood, and will determine your blood type. We shall examine also the anatomy of the circulatory system and the heart, and will observe the absorption spectrum of hemoglobin and its changes on combination with oxygen and carbon monoxide.

BLOOD CELLS

Swab the ball of your middle or forefinger with 70% alcohol. Using a *new*, *sterile* lancet, puncture the skin lightly, so that you can squeeze out a drop of blood. Touch this to a microscope slide about 1 cm from the end.

[At the same time suspend another drop or two of blood in about 5 drops of isotonic saline solution (0.9% sodium chloride in water) in a small test tube. This concentration of salt solu-

tion has the same osmotic pressure as the blood and will keep the blood cells in good condition. This suspension of blood cells will be used for blood typing.]

The drop of blood that has been placed on the slide should be spread evenly and *very thinly* by drawing it along the slide with the end of a second slide. When the film of blood has dried, cover it with a few drops of methyl alcohol, and let it stand for 2 or 3 minutes. Drain off the alcohol, and immerse the slide for 6 seconds in the red stain which has been provided. Then rinse in a gentle stream of tap water for a few seconds. Allow the slide to drain again, and immerse for 6 seconds in the blue stain. Rinse again in tap water, drain, and examine under the microscope.

Identify the various types of blood cells. Most common of course will be the erythrocytes, which appear red. The nuclei of the leucocytes stain blue; it should be possible to tell the difference between the lymphocytes and granulocytes by the shapes of their nuclei.

BLOOD GROUP TYPING: AN ANTIGEN-ANTIBODY REACTION

The entire human race can be divided into four categories on the basis of their blood types (A, B, AB, O). Erythrocytes may contain antigenic proteins on their surfaces, designated A or B, or AB, if both are present. If neither antigen is present, the letter O is used. Persons of the A type have in their serum an antibody known as anti-B, which specifically reacts with erythrocytes containing the antigen B. Similarly, persons of the B type have an antibody, anti-A, which reacts with A erythrocytes. Blood of type O contains both anti-A and anti-B antibodies, and AB blood contains neither of these antibodies.

If the blood from an A person is transferred into a B person the antibodies of the host serum react with the antigens of the donor's red blood cells and cause them to clump together or *agglutinate*. This blocks the blood vessels and may kill the person. It is the antigen more than the antibody of the donor which causes severe damage when injected into an incompatible person, since the bulk of the blood cells agglutinated are those of the donor. What happens when B blood is transfused into an A recipient?

Although blood-group typing is an example of an antibody-antigen reaction, it is unusual in that these antibodies are present in the circulatory system without having been stimulated by an invasion of foreign material. Usually antibodies are made only in response to the presence of a foreign antigen. Yet no blood group antigen need ever have been present in man for the blood group antibodies to develop.

Blood groups are determined genetically. They are distributed differently in the various human races. The distribution among white Americans is as shown in the table, where the plus sign indicates agglutination or clumping of cells.

To determine your blood grouping, use the suspension of cells that you prepared in normal saline solution. Draw a line across the middle of a microscope slide with a wax crayon. Place 1

Blood group	Blood cells agglutinated by:		% in White Americans
	Anti-A serum from Group B donors	Anti-B serum from Group A donors	
O	—	—	45
A	+	—	40
B	—	+	10
AB	+	+	5

drop of the cell suspension on each half of the slide. To the drop on one side, add a drop of anti-A serum, to which a blue dye has been added; and to the drop on the other side, add a drop of anti-B serum, which has been dyed yellow. (Be sure to mark which is which!) Mix the drops by jittering the slide gently for 15 to 20 seconds, being careful not to let the drops run into each other. Can you observe any agglutination? Place cover slips on the slide and examine both drops under the microscope. Compare with a drop of blood suspension to which no serum has been added. What is your blood group?

Recently a number of blood factors in addition to the A, B, AB and O groups have been discovered. Probably the most important of these is the Rh factor. About 85% of the white race and 99 to 100% of certain other groups (Chinese, Japanese, African Negroes, and North American Indians) have an antigen called the Rh factor in their red blood cells. (The abbreviation "Rh" refers to the Rhesus monkey in whose erythrocytes, as part of the cell membrane, this antigen was first discovered.) Blood containing the Rh factor is known as Rh-positive; that lacking it, Rh-negative. If blood from an Rh-positive person is transfused into an Rh-negative recipient, an antibody (anti-Rh factor) is produced. This in itself is not harmful, but if a second transfusion is given, the anti-Rh antibody which has accumulated in the recipient's blood reacts with the Rh antigen introduced with the new red blood cells, and the result is often fatal.

The anti-Rh antibody may also be produced in an Rh-negative woman who, having an Rh-positive husband, bears Rh-positive children. During pregnancy some fetal red blood cells containing the Rh antigen may leak into the mother's circulation and cause the formation of anti-Rh antibody. This has no ill consequences for the mother, unless she later receives a transfusion of Rh-positive blood. Usually also the first child is not seriously harmed, since the mother's antibody titer is still low. During later pregnancies, however, the mother's anti-Rh antibody may enter the fetal circulation and destroy the fetal blood cells. This condition, known as *erythroblastosis fetalis*, causes the death of the child, unless its blood can be replaced in a massive transfusion by Rh-positive blood free from the antibody.

The test to determine the presence of Rh factor in blood is tricky, and inaccurate results are often obtained unless great care is taken to standardize the procedure. For this reason we will not attempt it in this laboratory.

THE HEART

(Do not fail to read Wiggers's fine paper listed in the Readings. Bring it to the laboratory, if possible.)

The heart, which is a single, two-chambered organ in fishes, is a double organ in the birds and mammals, with a lung heart on the right and a body heart on the left. The right atrium (auricle) receives blood drained by the veins from the tissues throughout the body and passes it into the right ventricle, which pumps it to the lungs for gas exchange. The oxygenated blood is brought back to the left atrium, which passes it to the left ventricle, which sends it out through the arteries to the body tissues.

The heartbeat has an associated pattern. First the atria, their walls relaxed, distend with blood. Then the atria contract while the ventricles relax, transferring the blood to the ventricles. Then the ventricles contract, driving the blood to the lungs and tissues, the bicuspid and tricuspid valves preventing it from re-entering the atria. Similarly the semilunar valves prevent any suck-back of blood from the aorta and pulmonary artery during the next relaxation of the ventricle.

Body tissues → venae cavae → right atrium $\xrightarrow[\text{valve}]{\text{tricuspid}}$ right ventricle $\xrightarrow[\text{valve}]{\text{semilunar}}$ pulmonary artery → lungs → pulmonary vein → left atrium $\xrightarrow[\text{valve}]{\text{bicuspid}}$ left ventricle $\xrightarrow[\text{valve}]{\text{semilunar}}$ aorta → body tissues

Beef or sheep hearts will be available for examination. Identify the four chambers of the heart: the two large ventricles forming the tip, and the two smaller atria which lie above. Find the openings from the veins into the atria, and from the ventricles into the arteries. Examine the valves of the heart. The tricuspid valve is between the right atrium and ventricle, the bicuspid (or mitral) between the left atrium and ventricle. Between each ventricle and its artery, a semilunar valve is found. Try to imagine the action of each chamber and valve while the heart is beating and blood is flowing through it.

CIRCULATION OF BLOOD

After you understand the organization of the beef or sheep heart, two of you working together should obtain a pithed frog. Draw out the tongue, and pin it across the hole in the frog board, or pin out the webbing of one foot across this opening. (The tongue is usually more satisfactory because it has less pigmentation to obscure the capillaries.) Position the whole assembly on the stage of a compound microscope. You should be able to see clearly the circulating blood, and identify arteries (or arterioles), veins, and the interconnecting capillaries. Note the elasticity of the red blood cells as they course through the blood vessels. Can you find a leucocyte pushing through a capillary wall?

Slit open the abdomen of the frog to expose the beating heart. Trace the circulation through the lungs (pulmonary circulation). Find the aorta, the venae cavae, and the other major blood vessels. Take this opportunity to review the organs of the viscera.

Make a smear preparation of frog's blood on a microscope slide and stain it as you did your own blood. What striking difference do you see between human and frog blood cells?

Examine the prepared slides of an artery and a vein in cross section and of a piece of lung in cross section. Arterial walls are thicker and more rigid than those of veins, though both are composed of three layers. You will probably see red blood cells within the blood vessels.

HEMOGLOBIN

Most of the oxygen is transported in the blood stream in loose combination with hemoglobin. In vertebrates, the hemoglobin is entirely contained within the red blood cells. It is composed of a protein, globin, to which heme is attached as prosthetic group. Heme is a complex of ferrous iron (Fe^{++}) with protoporphyrin.

The function of hemoglobin depends upon its capacity to combine reversibly with oxygen: $Hb + O_2 \rightleftharpoons HbO_2$. Hemoglobin absorbs oxygen in the lungs, where the oxygen concentration is high, and gives it up again in the tissues, where the oxygen concentration is low.

Hemoglobin possesses a characteristic absorption spectrum, and each of its derivatives has a different spectrum. Much of the chemistry of hemoglobin has been learned by observing these spectra. We shall examine the spectra of hemoglobin and several typical derivatives with a hand spectroscope.

Begin by orienting yourself in the visible spectrum. See that it stretches from a wavelength of about 400 mμ, in the far violet, to 700 mμ, in the far red. The absorption spectra of hemoglobin and its derivatives are visible as shadows in the green and yellow regions. These are called *absorption bands*, and each is characterized by the wavelength at which the shadow is deepest, the *absorption maximum*.

Oxyhemoglobin

Examine a few milliliters of a diluted preparation of blood (1:100) in the hand spectroscope. Note two absorption bands in the green, at about 577 mμ and 539 mμ. These are the bands of oxyhemoglobin, HbO_2. Any hemoglobin exposed to air, as this is, is oxygenated.

Reduced hemoglobin

To the preparation you have just examined, add a minute amount of the reducing agent, sodium hydrosulfite (sodium dithionite, $Na_2S_2O_4$), and re-examine the spectrum. You

will now see the two former bands replaced by a single broad absorption band centering at about 565 mμ. This is the absorption band of reduced hemoglobin (Hb). The hemoglobin can be re-oxygenated by shaking vigorously; the former bands re-appear. This cycle can be repeated over and over again; indeed, this is how hemoglobin functions in the body.

Carboxyhemoglobin

A preparation of diluted blood through which carbon monoxide has been bubbled will be available. Examine its spectrum. Add sodium dithionite. What happens?

Spectrophotometers are available with which these absorption spectra can be accurately measured. Working in groups of 2 to 4, measure the absorption of each of the above solutions at 5 mμ intervals throughout the visible range (400 to 700 mμ). Plot the optical density against the wavelength.

Optical density or *extinction* is the most useful measure of absorption of light. If the intensity of light of a given wavelength entering a solution is I_0, and the intensity of light that emerges is I, then I/I_0 is the fraction transmitted, or transmittance. The fraction absorbed is $(1 - I/I_0)$. The optical density or extinction is $\log_{10} I_0/I$, i.e., the logarithm of 1/transmittance. It has the special virtue of being proportional to the concentration of pigment, and to the depth of layer. For example, on doubling either the concentration of hemoglobin or the depth of layer measured, one doubles its extinction at all wavelengths.

EQUIPMENT

Per student

lancet, sterile, disposable
microscope slides
cover slips
2 small test tubes

Per 2 students

frog
cork board
box of pins
compound microscope
dissecting microscope
slide of mammalian lung
slide of mammalian artery and vein

Per 8 students

bottle 70% ethanol
absorbent cotton
dropping bottle 0.9% sodium chloride solution
dropping bottle methyl alcohol
differential staining solutions for blood cells*
wax crayon
blood typing sera: anti-A and anti-B†
mammalian blood‡
carboxyhemoglobin solution: the carbon monoxide complex of hemoglobin is prepared easily by bubbling a gentle stream of carbon monoxide through defibrinated blood diluted 1:100 with water. This must be done in a hood!

Per laboratory

beef hearts
bottle sodium hydrosulfite (dithionite) $Na_2S_2O_4$
spectrophotometer (e.g., Bausch and Lomb "Spectronic 20")
hand spectroscope

*Red and blue stains as provided by Scientific Products Co. (Division of American Hospital Supply Corp., 1210 Leon Place, Evanston, Ill.; branch offices in many cities. Wright's stain may be used instead.

†Anti-A and anti-B serum can be obtained from most medical supply houses or from Hyland Laboratories, Los Angeles, Cal.

‡Fresh mammalian blood, most easily obtained from a slaughter house, can be defibrinated by shaking vigorously in a bottle with glass beads. Clotting can also be prevented by the addition of 0.1–0.2 ml 10% potassium oxalate for every 10 ml of blood.

XVII PERMEABILITY AND ACTIVE TRANSPORT: THE HAMSTER GUT

(Readings: Weisz, pp. 277–285. Villee, pp. 44–46; 299–303; 330–334. See also H. W. Smith, "The Kidney," *Sci. Am.* **188**, No. 1, 40–48, Jan. 1953, Reprint No. 37; and further discussion of the kidney in S.P.T., pp. 156–158 and in Weisz, pp. 459–462.)

Living organisms, plants and animals alike, are to a degree divided into compartments, separated from one another and from the external environment by membranes. The compartments may be cells, cell organelles, tissues, organs, or indeed entire multicellular organisms; but they have in common the fact that they are divided off from other compartments by membranes.

Each cell has its membrane. Each of such intracellular structures as the nucleus and mitochondrion has its own membrane. An entire tissue or group of tissues stretched between two spaces or bounding the surface of an organ may also function as a membrane. So, for example, the multi-tissued animals may be thought of as essentially saclike or tubular in construction, with an outer surface facing the external environment, and an inner surface surrounding the digestive cavity, both lined by membranes. Foodstuffs, waste products, salts, water, oxygen, and carbon dioxide—the continuous flow of material into and out of the organism that is a large part of its life—must all be transported through membranes.

This transfer takes place in various ways. Even the simplest biological membranes are *semipermeable*. They allow certain substances to pass through the membrane, while blocking the passage of others. In general, for water-soluble substances, this choice depends mainly on molecular size. The membrane acts as though it possessed pores of a certain effective size, which permit small enough molecules to go through and block the passage of larger molecules.

A second factor, added to semipermeability, is *selective permeability*. So, for example, cell membranes tend to pass fat-soluble molecules, almost regardless of size. So also many cell membranes tend to pass uncharged molecules much more readily than charged molecules; and many exercise a further selection by passing, for example, negative ions more readily than positive ions.

In the types of permeability so far mentioned, the driving force is the difference in concentration of the permeating ion or molecule on both sides of the membrane. Granted that the membrane permits a molecule to pass through it, the net diffusion is always from the more concentrated to the less concentrated side, and the

rate of diffusion is directly proportional to the difference in concentration on the two sides of the membrane.

A third factor regulating the penetration of substances through biological membranes is *active transport*. This is of the highest importance, and is the special subject of this week's experiment. In active transport, a specific mechanism exists, and work is done, to carry a substance through a biological membrane. Specificity and the expenditure of energy are the earmarks of this process. Diffusion is an energy-yielding process that can do work. Active transport is an energy-demanding process; work must be done upon it. Such active transport may take substances from a higher to a lower concentration, through a membrane that would otherwise block their passage. What is much more remarkable, active transport can take substances from a lower to a higher concentration, that is, against the concentration gradient, bringing them to many times the concentration they possess in the medium from which they are being absorbed.

Active transport can be thought of as a process of pumping. Little is know of the mechanism by which it occurs. On the other hand, it is clear that energy is required, and this is usually supplied as ATP. The specificity of the process is also apparent. Certain molecules may be passed by the membrane and concentrated, while very similar molecules are blocked. So, for example, many cells are able to concentrate L-amino acids, but not their "unnatural" D-amino acid isomers. These distinctions are frequently relative rather than absolute; the specificity frequently takes the form of a difference in *rate* of transport. So, for example, galactose, glucose, and fructose are all isomeric 6-carbon sugars ($C_6H_{12}O_6$), all of about the same size and shape. Yet a mammalian intestine absorbs galactose more rapidly than glucose, and glucose more rapidly than fructose. Similarly glucose penetrates the wall of the intestine much faster than such a 5-carbon sugar as xylose or arabinose, though the latter, being smaller, would diffuse faster through a semipermeable membrane. On the death of the cells lining the lumen of the intestine, all such distinctions are lost. Now all the hexoses penetrate at the same speed, and 5-carbon sugars faster than 6-carbon sugars.

We are going to study an example of the active transport of glucose between two compartments in the hamster: the inside or *lumen* of the small intestine, and the "outside," normally filled by the blood and lymph.

The absorption of foodstuffs in mammals takes place almost entirely in the small intestine. The *mucosa* lining the intestine is thrown into folds and ridges. Its surface is velvety with numerous tiny, fingerlike projections, the *villi*; and the individual cells lining the lumen have so-called *brush borders*, tiny projections of cytoplasm upon their outer surface, so that this also is velvety at another level of dimensions. All these devices increase enormously the absorbing surface of the intestine. In man it has been estimated that the total intestinal area effective for absorption is about 10 m^2. Compare this with the total area of skin, which is less than 2 m^2.

After passing through the intestine, food substances are absorbed into the blood vessels or lymph channels, and are transported first to the liver and then to other tissues throughout the body. The cells which cover the villi take an active part in transferring some of these substances from the lumen of the intestine (the mucosal side) to the outer space (the serosal side). If the transfer of such a substance as glucose were a matter of simple diffusion, the concentration on the serosal side would never become greater than that on the mucosal side. In fact, however, glucose is actively transported through the intestine, so that it can become several times more concentrated on the serosal than on the mucosal side.

The preparation and procedure we shall use for studying the active transport of molecules across the wall of the small intestine was devised by Dr. T. H. Wilson of the Harvard Medical School. The small intestine of the rat or hamster is removed and cut into sections a few centimeters long. These sections are everted (turned

inside out), filled with the solution to be studied, and tied off at both ends to form sausage-like sacs. The eversion places the serosal side toward the inside of the sac, and the mucosal side outward, so that the cells engaged in active transport can be kept supplied with oxygen. The sac is laid in the same solution it contains, and incubated with continuous aeration for some time. After incubation, the solutions inside and outside the sac are analyzed to reveal any change of concentration that may have occurred.

The hamster gut should be set up as described below as soon as you enter the laboratory. There are, however, several things that should be done during the 60 to 90 minutes of incubation. During this time the materials for the glucose analysis should be prepared. It would be wise to run through the analysis of the standard solution to make sure that everything is working well.

The hamster from which you removed the gut should also be used to review vertebrate anatomy. Trace the alimentary canal. Examine the prepared slides of intestinal tissues. Review also the circulatory system. Compare the hamster heart with that of the frog. Note the cheek pouches. What do you notice about the hamster stomach?

Try to find the major places in the body where substances pass from one "compartment" to the other. In which of these is one "compartment" the outside environment, or open to it? In this connection, do you regard the inner cavity of the gastrointestinal system as inside or outside the animal? How about the body cavity (coelom)? Does it possess an opening to the outside environment, not blocked by a membrane? in males? in females?

One of the principal organs for the exchange of dissolved substances is the kidney. Portions of the kidney provide prime examples both of diffusion through a semipermeable membrane, and active transport (cf. Homer Smith's article listed in the Readings). Examine the hamster kidney. Examine also the prepared slides, and identify Bowman's capsule, glomerulus, and tubules. Which substances are excreted by the kidney? Which retained? Where do these things occur, and what kinds of permeability are involved?

PROCEDURE

Students, working in pairs, will be given a freshly killed hamster. Slit open the belly so as to expose the viscera, being careful not to damage the intestine. Find the stomach. Snip off the upper end of the duodenum just below the stomach, and carefully uncoil the small intestine, using scissors to cut away the mesentary when necessary. When the lower end of the small intestine is reached, snip it loose, and place the entire intestine in a petri dish half filled with Krebs phosphate Ringer solution containing 20 millimolar (0.36%) glucose. Do not allow the intestine to dry!

All the remaining mesentery and fat should be stripped by hand from the gut. Cut the intestine into at least two (preferably three or four if your animal is large) 5- or 6-cm sections, beginning at the upper end. Using a dropper, gently force a little solution through the sections to wash out the contents.

The sections of gut are now ready to be everted and tied off.

The mucosal surface is extremely delicate and the success of your experiment depends upon it. Handle it as little and as gently as possible, taking special pains not to scrape or bruise it. Using the glass rods provided, push one end of the gut into the lumen until it appears at the opposite end. Complete the eversion by rolling the gut along the rod. Slip the gut off the rod, and immerse in fresh glucose-Ringer solution.

Tie a thread ligature tightly around one end. Fill a dropper with the glucose-Ringer solution and insert the dropper into the gut. Loosely knot a thread around the open end of the gut so that it can be tightened quickly. Force the solution from the dropper into the intestinal sac until the sac is completely filled but not grossly distended. Tighten the ligature quickly as the dropper is withdrawn.

Place the sac in a test tube containing 5.0 ml of the fresh glucose-Ringer solution, and leave it at

room temperature for 60 to 90 min (the longer, the better). Arrange for air to bubble through the solution during this time. This should be done gently so as not to mar the intestinal sac. Note the exact length of time that each sac is incubated.

Prepare the other sections of intestine exactly as above. One (or two) sacs should be incubated with aeration, as above, to allow metabolic processes to continue normally. The other sections of intestine are to have their metabolism stopped by adding an inhibitor of the respiratory production of ATP, dinitrophenol (DNP), and/or by stopping the aeration. To the tubes containing the sacs whose metabolism is to be inhibited, either add 1 drop of DNP solution, or stop aerating, or do both.

After the incubation is complete (60 to 90 min), remove the sac from the tube, blot it dry on a paper towel, and carefully cut open the end so as to collect the liquid inside in a small test tube or in the depression of a spot plate. This is a tricky operation, so use extreme care or you may lose the results of the experiment! Save the solution also in which the sac has been incubated.

Glucose analysis

Three drops of the solution to be tested are placed in a test tube, and 2 ml of Benedict's solution are added. Mix, and place in a boiling water bath for 5 min. The approximate concentration of glucose can be determined by comparison with known glucose standards run similarly. The following analyses should be carried out:

(1) solutions inside each of the intestinal sacs (4 tubes),

(2) solutions outside each of the intestinal sacs (4 tubes),

(3) the original Krebs phosphate Ringer solution with 20 m*M* glucose (1 tube),

(4) tubes containing 0, 1, 2, and 3 drops of the standard 50 m*M* glucose solution (4 tubes).

Be sure each tube is carefully labeled in such a way that the boiling water will not obliterate the label! Masking tape high up on the tube is convenient for this.

Benedict's reaction depends upon the reduction of blue cupric (Cu^{++}) to red cuprous (Cu^{+}) ions by the aldehyde group of the sugar. Cu_2O is the red product formed. The variation in color is a measure of the amount of glucose originally present. A clear blue solution indicates none; a deep red indicates a high concentration. (Recall a previous use of the Benedict test in Exercise IV, p. 23.)

Report your results as the approximate ratio of glucose inside the sac to that outside.

EQUIPMENT

Per 2 students

hamster (killed immediately before use, preferably without anesthesia, or by injecting a fatal dose of nembutal)
dissecting instruments
2 petri dishes
6 or 8 droppers (some or all should be long)
sewing cotton
glass rod (1.5 mm × 15 to 20 cm)
2 pipets (2 ml and 5 ml of solution have to be delivered)
18 test tubes
spot plate
paper towels
400-ml (or 250-ml) beaker for boiling water bath
bunsen burner
ring stand
metal gauze

Per laboratory

Benedict solution; prepare as directed in Exercise IV
Krebs phosphate Ringer solution
Stock solutions:
(1) 0.9% NaCl (0.154 *M*)
(2) 1.15% KCl (0.154 *M*)

(3) 1.22% $CaCl_2$ (0.11 *M*)
(4) 3.82% $MgSO_4 \cdot 7H_2O$ (0.154 *M*)
(5) phosphate buffer (0.1 *M*, pH 7.4) (17.8 gm of $Na_2HPO_4 \cdot 2H_2O$ + 20 ml NHCl diluted to 1 liter)

Use 100 parts of solution 1; 4 parts of solution 2; 3 parts of solution 3; 1 part of solution 4; 20 parts of solution 5. Add glucose to make 20-m*M* (0.36%) and 50-m*M* (0.90%) solutions just before using. The solution should be stirred while adding the phosphate to prevent precipitation.

dinitrophenol, saturated solution

prepared slides of intestine

prepared slides of kidney

large water baths at 100°C may be used instead of individual ones

XVIII THE NERVE IMPULSE*

(Readings: B. Katz, "The Nerve Impulse," *Sci. Am.* 187, No. 5, 55–64, Nov. 1952, Reprint No. 20. This excellent article contains what you really need to know. Read also R. D. Keynes, "The Nerve Impulse and the Squid," *Sci. Am.* 199, No. 6, Dec. 1958, Reprint No. 58; Weisz, pp. 475–480; Villee, pp. 354–358.)

One of the most important aspects of animal evolution is the development of systems of rapid intercommunication through nerve cells. Even some of the protists, notably the ciliates, possess intercommunicating systems of fibrillae that seem to help to integrate the cell's motions, and may be thought of as a sub-cellular nervous system. The coelenterates possess a diffuse nerve net. All the higher animals possess nervous systems made up of discrete, intercommunicating nerve cells.

One nerve cell meets another at a boundary called a *synapse*. Though a nerve cell conducts impulses equally well in both directions, a synapse transmits impulses only in one direction. It is this characteristic that limits nervous transmission to particular pathways and particular lines of flow. In general we distinguish excitations flowing into the central nervous system from the receptors (afferent) from excitations flowing out of the central nervous system (efferent) toward the effectors (the muscles and glands).

A nerve cell consists of a *cell body* containing the nucleus and its cytoplasm, from which springs a specialized, threadlike, conducting element, the *nerve fiber* or *axon*. In a higher animal the nerve cell bodies are all inside or just beside the central nervous system—the brain and central nerve cord. The nerves that one finds roaming about the body are bundles of axons.

The business of a nerve axon is to conduct an excitation. If we dissect a nerve out of the body together with the muscle that it innervates (for example, the frog sciatic nerve and the gastrocnemius muscle), we can stimulate one end of the nerve and know that it has transmitted an excitation by the fact that a moment later the muscle contracts. As the nerve conducts its excitation, sufficiently delicate instruments can measure the passage of an electrical change. This electrical change, which invariably accompanies the nerve response, is the *nerve impulse*, *action potential*, or *action current*.

An electric current is a flow of electrons from a region in which they are more concentrated to a region of lower concentration: i.e., from a more negatively to a more positively charged region. By an odd historical convention, the

* The instructor should consult Appendix B for information about the electronic equipment.

electric current is usually stated to be flowing just the other way, from positive to negative. You will have to remember, while using this convention, that the positive pole is where there are fewest electrons, and that when a current flows, the electrons flow *toward* the positive pole.

In any flow of current we must take account of three factors:

(1) An intensity factor, the *potential*, measured in volts. This is the *pressure* of electrons to flow from the region of higher to that of lower concentration of electrons. It is just like the pressure of water to flow from a higher to a lower level. Just as with water, one could measure this pressure without allowing any flow. It is this pressure of electrons that we measure as the electrical potential or voltage.

(2) A quantity factor, the *current* or *amount* of flow, measured in amperes. There is a certain pressure in the water mains, whether or not you use any water. You can then turn on a tap and allow the water to flow gently or strongly. The same is true of an electric current.

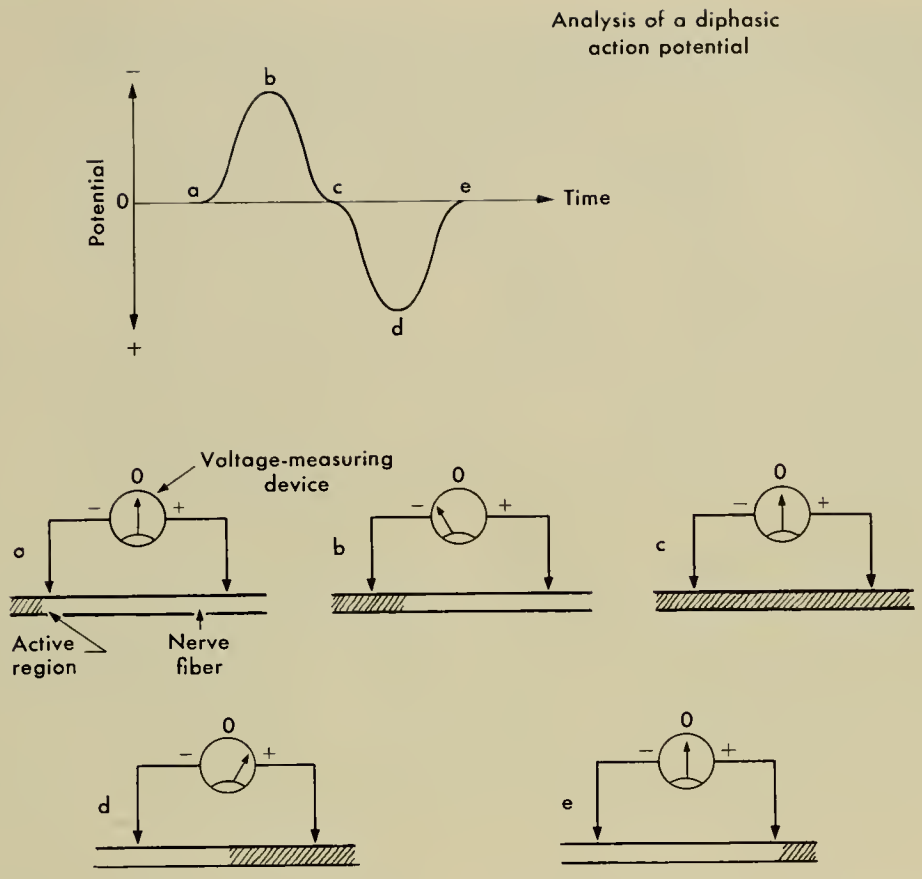
(3) The third factor is the *resistance* offered by the conductor, measured in ohms. To follow out the water analogy, one might have a narrow pipe, which even when entirely open allows the water to flow through it only slowly, or a wide pipe, which can conduct it very rapidly. Similarly a thin copper wire offers considerable resistance to the flow of current compared with a thick wire. In these cases the potential may be identical; but the flow of current is very different.

These three quantities are bound together in the simple relation expressed in Ohm's Law: $E = IR$, in which E is the potential (volts), I is the current (amperes), and R is the resistance (ohms).

If a very fine electrode is inserted into the interior of a nerve fiber, and another electrode touches its surface, one finds a more-or-less constant electrical potential between these two electrodes, called the *resting potential*. Such an experiment is best done on the giant nerve fibers of the squid (see Keynes's article). This resting potential is about 75 to 90 millivolts (mv), with the outside of the nerve fiber positive to the inside, the nerve membrane forming the intervening boundary. The source of the potential is a differential distribution of ions: more K^+ ions inside, and more Na^+ and Cl^- ions outside. The selective permeability of the nerve membrane, which is largely responsible for this differential distribution of ions, is spoken of as its *polarization*.

A nerve impulse results from a local *depolarization* of the membrane, permitting ions to flow through it more freely. The active point on the nerve fiber has momentarily ceased to maintain the differential distribution of ions just described. As a result, the surface of the cell at this point has lost its special positivity; it is therefore negative relative to the remaining cell surface. This change is *self-propagating*, each such active point stimulating the adjacent region of the fiber. For this reason the nerve impulse appears as a wave of negativity that sweeps down the length of the nerve fiber. It is important to realize that when a nerve fiber carries an impulse, all that it conducts is this excitation. There is no actual flow of either energy or material from one end of the fiber to the other; there is only the passage of excitation.

This point can be made clear by a simple example. The conduction of a nerve impulse is not like the conduction of water through a pipe, or of electricity through a wire. It is like the flash that passes down a train of gunpowder if we ignite one end. If you will think of such a train of gunpowder, you will understand readily many important properties of the nerve impulse. So, for example, it is obvious that at each point the gunpowder generates its own response, independently of all the other points. You would get the same response whether you lit one end with a spark or a bonfire. Where there is a lot of gun powder, there will be a big response; where the gunpowder is thinly sprinkled, there will be a



weak response. At each point, however, the gunpowder will give everything it has. That is, the response will vary in strength from point to point with the amount of gunpowder, but at each point it will be *all or nothing*. In the same way, and for much the same reasons, the response of a nerve fiber is independent of the strength of the stimulus, provided it is strong enough to excite at all; and is also all or nothing.

We measure nerve response by placing two electrodes on the nerve and connecting them through a sufficiently sensitive recording device. The quantity measured is the *potential*. (The nerve membrane has a very high resistance, so that the current flow is very small.) The two electrodes, touching the outside of the fiber, normally have zero potential between them. If we now excite the fiber, as the nerve impulse comes under the first electrode that electrode becomes negative to the other electrode. The instrument records that negativity. As the impulse sweeps on, it may come to involve equally both electrodes. In that case there is momentarily again no difference of potential between the two electrodes. Then the nerve impulse has passed the first electrode and involves only the second, which now momentarily becomes negative to the first; the recorded potential is now just opposite to what it was before. Then the impulse is past, and again, as at rest, there is zero potential between the electrodes.

The result is a so-called *diphasic* change of potential: the potential starts at zero, sweeps up to a maximum in one polarity, comes down again through zero to a maximum in the opposite polarity, and returns to zero. These changes should be obvious from the accompanying diagram.

All this discussion has been in terms of a single nerve fiber. Nowadays physiologists often work with such single nerve fibers, either the giant nerve fiber of the squid or with the very much smaller nerve fibers of higher animals including mammals. For the latter one needs to use microelectrodes, which may be only 1μ in diameter.

You must remember that the nerves that you see in animals and with which you will be working are *bundles* of many such nerve fibers. Though each individual nerve fiber exhibits all-or-nothing behavior, the nerve bundle does not, since a strong stimulus may excite many nerve fibers whereas a weaker stimulus may excite few. Many other characteristics of the nerve impulse, however, can be studied in such nerve bundles.

In order to measure nerve impulses, very sensitive devices must be used. At the present time all such work is done with electronic amplifiers and recorded with oscilloscopes. The expense and complexity of such equipment has in the past restricted neurophysiological experimentation to relatively few places and relatively advanced courses. This is a great pity, for the phenomena are of the highest importance, generality, and interest. The experiments we are about to describe involve the use of the equipment described in Appendix B. It is the finest equipment of its kind that is available, quite adequate for advanced research in neurophysiology, yet designed for maximum dependability and simplicity of operation. Fundamentally it is not very much harder to use than a television set. We hope that it will be a relief to you to see what electronics can do when it is not producing soap operas.

In this first period, working in groups of four, everyone should learn to operate the electronic equipment and set up and record the impulses from a frog sciatic nerve. The best arrangement is for two of you to begin at once to dissect out the frog sciatic nerve, while the other two learn how to operate the equipment. Then, after the preparation is set up, the students who know how to work the apparatus can teach the others how to do it while recording from the nerve.

THE ELECTRONIC EQUIPMENT

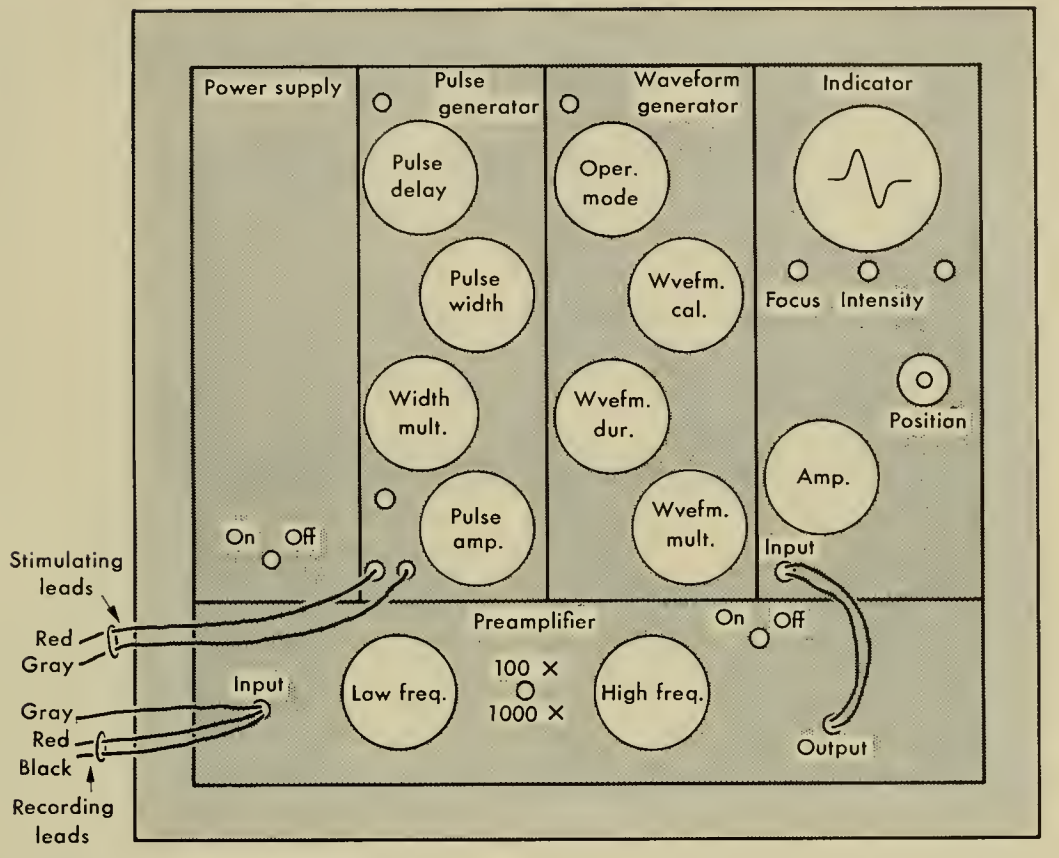
This equipment may at first glance seem complex and forbidding. It is, in fact, simpler than it looks. Many of the dials are simply multipliers of the same adjustment, or of no concern in the normal operation of the instrument. There are only five or six controls you must learn to operate.

The general principle for study of such small electrical changes as found in nerve cells is first to amplify the potentials and then to observe their magnitude, duration, and shape by means of a recording device. From the nerve chamber, which will enclose the preparation, note the leads into the preamplifier. We shall speak of the nerve potential and its changes as the *signal.* The preamplifier amplifies this signal 100 or 1000 times. The other dials on the amplifier allow one to select certain frequencies of signal while eliminating others. These need not concern us today, but next week we will have opportunity to use these controls.

To observe and measure the response, the magnified signal is led into the *indicator*, an oscilloscope with its amplifier. In the indicator, the impact of a narrow beam of electrons on a fluorescent screen makes a spot of light, as in a TV set. The beam of electrons on its way to the screen passes between two metal plates, charged with the amplified biological potential. The beam of electrons, themselves negatively charged, is deflected toward the positively charged plate and away from the negatively charged plate, so displacing the spot of light on the screen from the zero position which it occupies when both plates are equally charged. In this way the polarity and magnitude of the signal are reflected in the direction and extent of the deflection of the spot of light on the screen.

The controls on the indicator (*intensity*, *focus*, *position*, etc.) are familiar to anyone who has ever adjusted a TV set. The calibrated amplifier in the indicator allows further magnification or attenuation of the input signal.

Besides polarity and amplitude, we are interested also in the change of biological potential

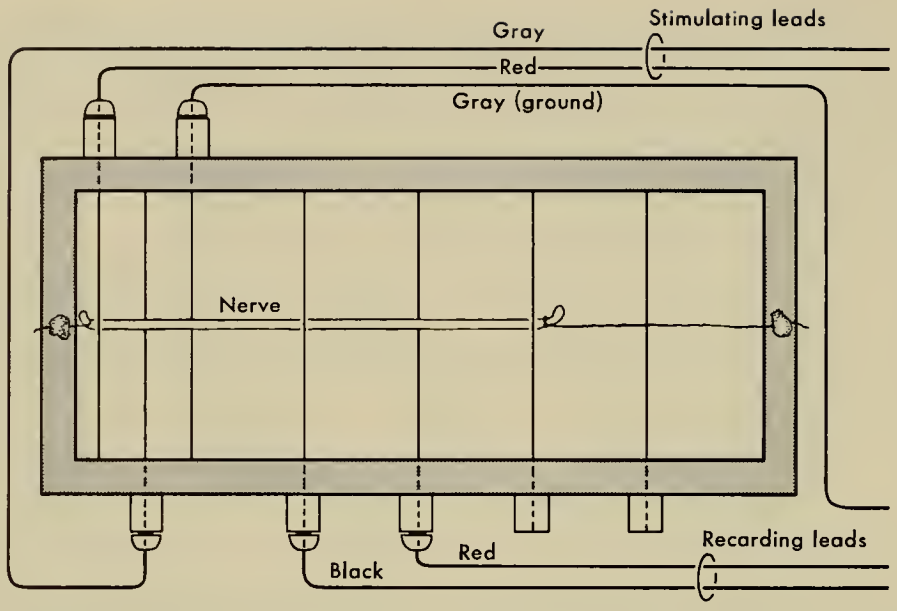


with time. To study this, the signals are applied to the electron beam while it is moving horizontally across the screen at some prescribed rate. The horizontal sweeping of the electron beam is controlled by the *waveform generator*, which sets high voltage potentials across a pair of plates that deflect the beam in the horizontal direction. We control the *rate* of the horizontal sweep by the *waveform duration* dial. The *operating mode* dial lets us select how the sweep is to be controlled, whether automatically or manually. (Ignore the "gated" and "triggered" selections on the equipment; these are for special applications.)

These instruments and the power supply complete the recording setup. However, to study nerve impulses, we must stimulate the nerve to fire. This can be done artificially by applying a brief electrical shock. (Next time we shall study the biological initiation of nerve impulses by light striking a photoreceptor.)

The *pulse generator* provides controlled electrical stimuli. It, like the indicator sweep, is activated by the waveform generator. Thus for every sweep of the indicator beam, one stimulus pulse is delivered. The pulse generator has three controls: the *pulse delay* knob sets the point during the horizontal sweep at which the stimulus pulse is delivered; the *pulse width* knobs control the duration of the stimulus pulse; and, finally, the *pulse amplitude* controls its *voltage*.

That is all there is to the equipment. To familiarize yourself with the controls, connect the lead from the "pulse out" terminal of the pulse generator to the "vertical input" of the indicator. (The pulse generator provides enough voltage so that the preamplification can be



omitted.) Convenient settings to begin with are:

Indicator:
"5 volts/division" amplification;
Waveform generator:
"recurrent operating mode,"
100 msec "waveform duration";
Pulse generator:
0.3 "pulse delay,"
10 msec "pulse width,"
0 "pulse amplitude."

Turn the instruments on and allow a few minutes for them to warm up. Focus, center, and brighten the beam if necessary. With the "waveform duration" dial of the waveform generator, alter the rate of horizontal sweeping of the electron beam. A setting of 100 msec means that it takes 100 msec (0.1 sec) for the electron beam to complete a sweep. Then, with the wave duration set at 100 msec, *very slowly* turn up the pulse amplitude of the pulse generator, and observe the square wave deflection on the oscilloscope screen. (Check that the pulse amplitude you are putting in is giving a deflection that agrees with the indicator amplifier.) Now vary all the controls mentioned above, and become familiar with them. The only caution is not to put so much pulse amplitude voltage into the indicator that the beam is deflected off the screen; this might cause damage.

NERVE PREPARATION

After pithing the frog, strip the skin off one leg. Lay the animal on the dissecting pan, belly down, and expose the sciatic nerve. With glass needles, free as much of the nerve as you can (about 4 cm) from the surrounding tissues, being careful not to stretch or injure it. The more nerve you expose, the better for your experiment. Keep the nerve wet with Ringer solution throughout the dissection. To expose the upper part of the nerve, you will have to remove the overlying pelvic girdle. When the nerve is freed from the surrounding tissues, tie a short length of thread to each end; *measure the distance between the threads.* Cut beyond the threads, and remove the nerve. Keep track of which end is proximal and which distal.

Mount the nerve in the nerve chamber so that each end passes underneath an electrode, while the middle section weaves over and under the intervening electrodes. (See diagram.) The

proximal end of the nerve should be in contact with the three closely spaced electrodes used for stimulation. Extend the nerve to its *in situ* length in the chamber; *do not overstretch it*. Fix the ends of the threads to the edge of the chamber with plasticene. Pour mineral oil into the chamber to cover the nerve. The oil insulates the nerve and prevents it from drying out.

EXPERIMENTAL PROCEDURE

Note: Before connecting any leads to the nerve chamber, make sure that the pulse amplitude dial on the pulse generator is turned to 0. If you accidentally put high voltages through the nerve, it will be ruined very quickly.

Two leads (gray and red) from the pulse generator should be connected to the first two, closely spaced, stimulating electrodes; and two leads (red and black) from the first two recording electrodes to the input plug of the amplifier. The gray ground lead should be connected to the third, closely spaced electrode. (See diagram.) Use the following settings:

Preamplifier amplification	= 100
Indicator amplifier	= 0.5 volts/division
Waveform duration	= 10 msec
Pulse width	= 0.1 msec
Pulse delay	= 0.3
Pulse amplitude	= 0
Pulse	= negative

Now *very slowly* increase the pulse amplitude. Note first the stimulus artifact, and then the appearance of the nerve action potential. Increase the pulse amplitude until the nerve impulse is maximal in height.

The nerve impulse should appear as a diphasic wave. Why? What is its maximal voltage? What is its duration? Make a graph of voltage vs. time. Calculate the latency of the nerve impulse by measuring the distance from the start of the stimulus artifact to the beginning of the response (the stimulus artifact is conducted down the outside of the nerve and is recorded instantaneously). Most of the latency is occupied by the time it takes for the nerve impulse to travel from the stimulating electrode to the first recording electrode. Estimate the speed of conduction of the main group of sciatic nerve fibers from the latency and the distance between these electrodes. Express this velocity in meters per second.

Determine the minimal stimulus voltage needed to evoke minimal (threshold) and maximal responses. Why can't you increase the amplitude of the nerve potential indefinitely by increasing the stimulus? How does the observed grading of the response fit in with the all-or-none law? Decrease the duration of the stimulus (pulse width) and redetermine the voltages that produce minimal and maximal responses. Try several different pulse widths. Make a plot of pulse duration vs. threshold voltage. What do you conclude?

Next, crush the distal end of the nerve with a fine pair of metal forceps just before it passes underneath the most distal electrode, and reconnect the recording leads to the two most distal electrodes (one on each side of the crushed portion). Observe that the nerve impulse is now monophasic. Why?

There are several classes of fibers in the frog sciatic nerve which conduct impulses at different rates. With monophasic recording you may notice humps on the down sweep of the nerve impulse. These represent the slower conducting α_2, β, or γ fibers separating from the prominent and most rapidly conducting α_1 fibers. To see these humps clearly, set the "operating mode" to manual, and trigger the impulses by hand at a rate of about 1 per second. Try to estimate the rates of conduction of each of the fiber classes. See if the groups of fibers differ in threshold, by determining the pulse amplitude needed to produce a maximal response in each group.

Finally, determine the maximum frequency with which the nerve can respond. This is done by progressively decreasing the "waveform duration," so decreasing the intervals between successive stimuli. At what frequency of stimulation do the responses begin to decline in amplitude? What can you conclude concerning the *refractory period* (see Katz's article)?

ACTION POTENTIALS OF HUMAN NERVE AND MUSCLE

It is possible to record indirectly the nerve and muscle action potentials in your own hand by means of remote electrodes when the hand is in a nonconducting medium such as distilled water. For this experiment, fill a porcelain pan with distilled water and suspend about 6 inches of the two heavy copper wire electrodes in the water at the two ends of the pan with clamps. Make sure the exposed ends of the electrodes are entirely under the surface of the water. Connect the leads from the electrodes to the binding post terminals on the side of the copper cage, and connect the input leads from the amplifier to these terminals. Use the following settings:

Amplification = 1000
Indicator amplifier = 0.05 volts/division
Waveform duration = 1000 msec

Hold your hand relaxed in the water. When the baseline has settled down to a steady trace, clench your fist. Individual action potentials of 200 to 300 μv should be seen. Remember that in the frog nerve, you were stimulating all the nerve fibers simultaneously, so that it appeared as if you were eliciting just one large action potential. Here the action potentials in the various fibers (both nerve and muscle) are staggered in time, and consequently appear as smaller single spikes. To see the single action potentials more clearly, increase the sweep speed by decreasing the waveform duration to 100 msec. Note that the action potentials continue as long as the fist is clenched. One nerve impulse serves only to twitch a muscle. For a muscle to remain contracted, it must receive a continuous train of impulses. Alternately clench and relax your fist as quickly as you can; note the bursts of impulses that excite each tightening of the fist.

From this preparation, you can also learn something about the anatomy of the hand. Many of the movements of the hand, such as flexion and extension of the fingers are performed by muscles in the forearm which connect to the fingers by tendons. Flex your fingers under the water and note that you see no action potentials. Try spreading your fingers sideways, and bringing them together again. What do you conclude? Move your thumb and hand in as many ways as possible and try to decide which movements are performed by the muscles in the hand and which by the muscles in the forearm. Check your conclusions in an anatomy atlas.

EQUIPMENT

Per 2–4 students

electronic recording and stimulating setups
frog
dissecting pan
goose-neck lamp
thread (1 spool)
plasticene
beaker (400 ml)
medicine dropper
plastic ruler
nerve chamber
2 glass dissecting needles
mineral oil (to fill nerve chamber)
frog ringers (25 ml)
porcelain pan (approx. 12″ × 8″ × 8″)
2 heavy copper electrodes (heavy copper wire flattened about 6″ at one end and otherwise insulated works very well)
2 electrode clamps
distilled water

XIX MUSCLE

(Readings: Weisz, pp. 356–362; 446–457. Villee, pp. 48–50; 344–351. H. E. Huxley, "The Contraction of Muscle," *Sci. Am.* **199**, No. 5, 66–82, Nov. 1958, Reprint No. 19. C. J. Wiggers, "The Heart," *Sci. Am.* **196**, No. 5, 74–87, May 1957, Reprint No. 62.)

The ability to move rapidly is one of the major characteristics of animal life. In all but the lowest animals, such motions are accomplished by muscles. Throughout the animal kingdom, muscle tissue is built upon a common plan, and is remarkably uniform.

During the past few years we have begun to learn how muscle works. Upon excitation of a muscle, through a nerve or by the same artificial devices one uses to excite nerves, a wave of depolarization much like the nerve impulse passes down the muscle membrane. Somehow this releases ATP, which reacts with the proteins, actin and myosin, of which the muscle fibrils are mainly composed, causing the muscle to contract. In this process, ATP is broken down to ADP, yielding with the release of its high-energy phosphate bond the chemical energy that is converted in the muscle contraction to mechanical work. Actin and myosin are long, fiber proteins, arranged in alternate, overlapping sequences along the muscle fiber. During contraction, the actin and myosin filaments slide over one another so that they overlap further, causing shortening (see Huxley).

How ATP causes these changes is not known; nor do we know how the depolarization of the muscle cell membrane excites this reaction. We do know, however, that the wave of excitation that passes over a muscle fiber on stimulation is very much like the nerve impulse.

Three types of muscle tissue are found in vertebrates: striated, cardiac, and smooth muscle. The rapidly contracting "voluntary" muscles of our arms, legs, and trunk are striated. The cardiac muscle that forms the wall of the heart, is also striated, but is otherwise intermediate in structure and speed of contraction. Smooth muscle, found in the gut and blood vessels, undergoes slow, sustained contractions, as for example the slow peristaltic motions of the intestine. All three types of muscle, though histologically and functionally distinct, owe their contractility to actin and myosin.

Since they do contain the same contractile proteins, one might wonder why these different types of muscle possess such different properties. One reason is large differences in structural arrangement, apparent in part under the microscope, and persisting down to the molecular level. Another factor is that the cell membranes of the various types of muscle, as also of nerve, have very different excitatory characteristics. So, for example, the larger frog nerves conduct

impulses at about 30 m/sec. Striated muscle conducts at only 2 to 5 m/sec; heart muscle at less than 1 m/sec, and smooth muscle at only 5 to 20 cm/sec. The more slowly the excitatory impulse is conducted over a muscle fiber, the more slowly it responds.

The excitability of such cell membranes can be drastically altered by chemical reagents. Nerve cells transmit their excitation across a synapse with another nerve or with a muscle by releasing at the synapse excitatory or inhibitory substances (neurohumors, "nerve hormones"). The usual excitatory neurohumor is acetylcholine, though at the first line of synapses in the sympathetic nervous system it is epinephrine (adrenaline). In such cases the neurohumor released by an excited nerve cell at the synapse locally depolarizes the membrane of the succeeding nerve or muscle cell, so exciting it in turn.

In addition to such substances which depolarize nerve and muscle cell membranes, exciting them or lowering their thresholds to other stimuli, there are also *hyperpolarizing* substances, which *increase* the polarization of the membrane, raising the threshold of the cell and making it more difficult to excite. These are therefore not excitatory, but inhibitory substances, and may be released at nerve endings which are concerned with inhibition, rather than with excitation. γ-amino butyric acid,

$$(CH_2NH_2 \cdot CH_2 \cdot CH_2 \cdot COOH),$$

acts in this way; but, as we shall see, acetylcholine also may at times, as in the heart, inhibit rather than excite.

The characteristics of the excitable membranes of nerves and muscles can be altered and controlled by the application of such substances, released naturally at nerve endings, or applied artificially. The rate of the heart beat, for example, as well as its amplitude, are controlled in this way. The heart muscle beats automatically, as a result of an intrinsic cycle of excitation and recovery. The heart rate is regulated through the activity of two nerves, which are opposed in their effects: a nerve from the sympathetic system, which releases epinephrine at its synapse with the heart muscle and excites the heart to beat more quickly; and the vagus nerve from the parasympathetic nervous system, which inhibits the heart by releasing acetylcholine, slowing the beat.

During this period we shall examine the structure of the various types of muscle, the contractile effects of ATP upon the muscle proteins, and the effects of excitatory and inhibitory substances on the frog heart.

EXPERIMENTAL PROCEDURE

Types of muscle

First examine the prepared sections of striated, smooth, and cardiac muscle. Striated and cardiac muscle may be recognized by their cross-banding or striations. Smooth muscle has no visible banding. Each of the long, spindle-shaped cells of smooth muscle contains a prominent, elongated nucleus. The fibers of striated muscle seen under the microscope are distinct cells, lying parallel to one another. The similarly striated fibers of cardiac muscle, however, branch widely with one another, forming an intercommunicating network that contains many nuclei, but no apparent cell boundaries. Until recently the entire ventricle of the heart was thought to constitute a single, multinucleate cell (a so-called syncitium). Recently, however, membranes that divide cardiac muscle cells have been found with the electron microscope.

Using Huxley's article as guide, study the banding of a microscopic section of striated muscle. Identify the *A* and *I* bands and the *Z* and *H* lines. What arrangement of molecules accounts for the *A* and *I* bands?

Contraction of glycerinated muscle

Next, we shall study the contraction of muscle fibers on addition of ATP, using the famous and extraordinarily important preparation devised by Albert Szent-Györgyi. The psoas muscle of a rabbit contains exceedingly little connective tissue, being composed almost entirely of long, parallel muscle fibers. It is the lack of connective tissue that makes this the "tenderloin." A

rabbit had been killed, hastily eviscerated, and the body wall chilled. The psoas muscles lie at both sides over the backbone. They are readily divided into narrow strips, and each such strip was tied at its ends to an applicator stick at the slightly stretched length at which it was found in the body. Then the muscle strips, tied to their sticks, were cut away, and soaked in a half-and-half mixture of glycerol and water at 0°C for two weeks or longer.

This procedure removes almost all the contents of the muscle fibers except for the contractile proteins, actin and myosin. The muscle still retains its striated appearance under the microscope, and can still contract when supplied with ATP. Indeed it retains these properties for many months in the cold glycerol solution.

(The following experiment should be completed during the first hour of the laboratory.)

One such strip of glycerinated muscle will be given to each group of four students. With scissors cut the muscle just inside the sutures (knotted threads) that hold it to the stick, so as to get as long a piece as possible, and then cut this in half, so that each pair of students gets half the strip. Drop each piece into a small petri dish containing cold glycerol-water mixture.

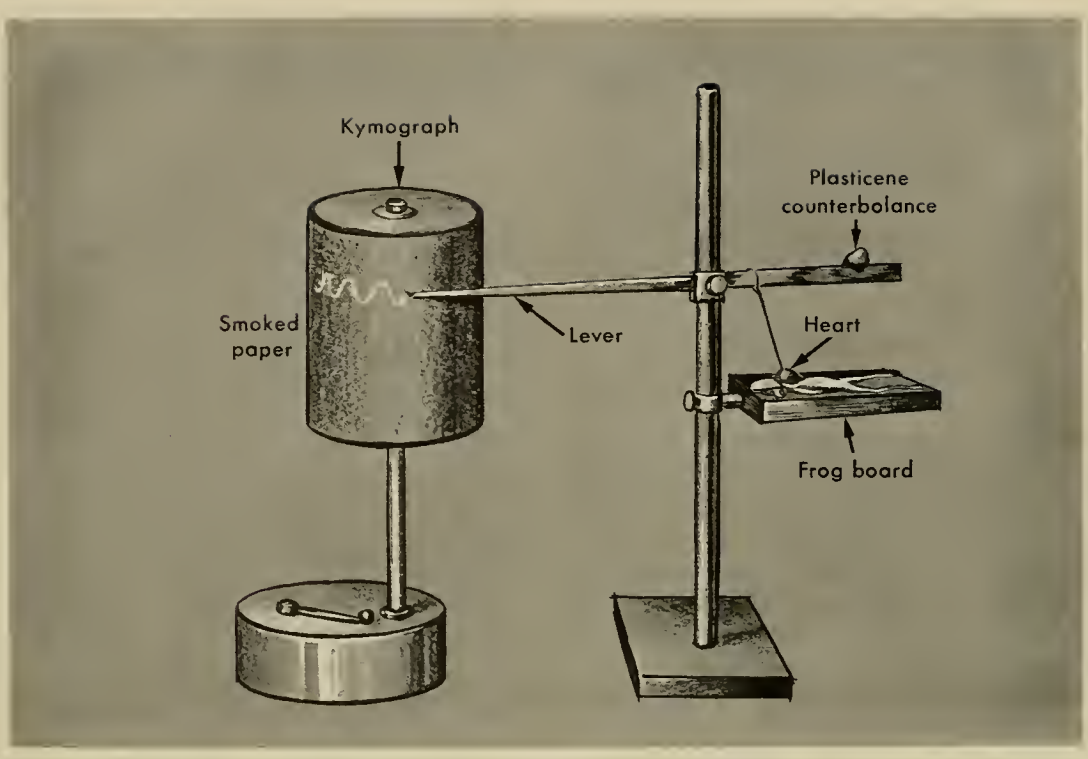
With fine forceps and dissecting needles, tease out narrow threads of muscle about 15 mm long and about as thick as silk thread. Place one of these in a drop of cold glycerol-water mixture on a microscope slide, and examine its structure in the microscope. Can you see the striations? (To see them clearly, you may have to separate out a single fiber.) Transfer the slide to the stage of a dissecting microscope, and measure the length of the muscle with a ruler. Add a drop of 0.25% ATP solution containing 0.05*M* KCl and 0.001 *M* $MgCl_2$. Remeasure the length of the muscle, and re-examine its striations in the compound microscope. (If the strip curls during contraction, it is too thick.) Can you distinguish any differences?

Try this several times, each time measuring the original and final length of the fibers. Record your results. Does a second drop of the ATP-salt solution have any further effect? For contraction to occur, certain ions must be present in specific proportions, in addition to ATP. To a degree, other ions can be substituted for the K^+ and Mg^{++} that we use here, but no other substance seems to substitute for ATP, which seems specific for this process. Convince yourself of the need for the ions and the ATP by adding drops of ATP alone, and KCl-$MgCl_2$ mixture alone, to narrow threads of muscle.

Hormonal control of the frog heart

By the end of the hour, turn to the next experiment, on the effects of acetylcholine and epinephrine (adrenaline) on the frog heart. Work in pairs. While one partner is preparing the animal, the other should become familiar with the operation of the kymograph. This will be demonstrated by the instructor. A revolving drum has a piece of smoked paper wrapped around it. (Be careful not to touch or otherwise smudge the paper!) The speed of the drum is controlled with a knob on the base.

Expose the heart of a pithed frog, freeing it from the pericardial membranes, and cutting away the body wall over it. Grasp the tip of the ventricle with a pair of fine forceps, and pass a thread through it with a needle and thread. Tie it securely (this is called a suture in surgery), but not so tightly as to cut the tissue. Keep the heart continuously wet with fresh Ringer solution. Place the frog on the frog board, with the heart directly beneath the kymograph lever, and attach the other end of the suture to the lever with a bit of modeling clay. The heart should extend well out of the chest cavity and the lever should be about parallel with the table. If it seems to strain the heart unduly to lift the lever, help to balance the lever with a little lump of clay at the end attached to the heart. The lever should now be moving up and down rhythmically with the heartbeat. Move the kymograph so that the tip of the lever just touches the smoked paper, and the lever is tangential to the drum. (See diagram.) Make sure you start your record high up on the drum, so that you can get another record below it.



Keep dripping Ringer solution on the heart; if you let it dry, the amplitude of contraction will decline. When the heart is beating evenly, rotate the drum a little way so as to draw a control record of 6 to 8 beats. Rinse the heart with 2 to 3 drops of Ringer, wait 30 seconds, and repeat. There should be no difference in the two records. (Writing on the smoked paper with your dissecting needle, indicate on the record when you add anything to the heart.)

Now rinse the heart with 2 (only 2!) drops of acetylcholine solution in Ringer. Watch the heart, and when you see a perceptible change of beat, run a short record. (If nothing happens within 2 minutes, add 2 more drops of the acetylcholine solution, but no more. Too much acetylcholine will cause the heart to stop completely. If your heart does stop from too much acetylcholine, rinse it thoroughly with Ringer solution, and it should recover within about 5 minutes.)

What has happened to the heart rate and the amplitude of contraction? After waiting another minute or so, run another record. Has the heart begun to recover? Now rinse the heart with fresh Ringer. The beat should return to normal in a few minutes.

Run another control record. Now rinse with 2 drops of epinephrine solution in Ringer. What does it do to the heart beat? Now rinse with 2 drops of acetylcholine solution in Ringer, and again record the result.

Again rinse the heart with Ringer, and wait for about 5 minutes. The heart may still show some effects of the epinephrine, which wear off much more slowly than those of acetylcholine. Run another control record. Add 5 drops of atropine solution in Ringer to the heart, wait for 30 seconds, and run a record. Has anything happened? Now add 2 to 4 drops of acetylcholine solution, and record again. What has happened? What did the atropine do?

The heart is self-excitatory. Its beat originates in a small specialized area in the wall of the sinus venosus, the SV node. In this area, the membrane allows a continuous, small leakage of Na^+

into the cells, so depolarizing them and causing them to fire impulses regularly. Acetylcholine exerts its effect only on this area, apparently by increasing the permeability of the cells to K^+, so allowing some K^+ to leak out. This hyperpolarizes the cells, and lengthens the time required for the sodium leak to depolarize them to the point of firing.

Epinephrine exerts its effect both at the SV node and over the entire heart, by increasing the cell permeability to Na^+, thereby tending to depolarize the membranes, and facilitating the conduction of impulses. Epinephrine also seems to have some effect on the contractile process itself, since it increases the amplitude of contraction of the heart fibers. Did you see this effect?

Atropine is thought to exert its effect by combining with the same sites on the membrane that acetylcholine would otherwise combine with, much as an inhibitor, by combining with an enzyme, blocks the substrate.

Finally, show that the ventricular beat originates in the atria by tying a suture (a thread) around the heart between the atria and ventricle. This pinches the specialized connecting cells which transmit the excitation from the atria to the ventricle. These cells normally delay the impulse long enough for the atria to complete their contractions before the ventricle begins to contract. When pinched by the thread, they may not conduct at all, or may conduct so slowly that they transmit only one impulse for every two or three contractions of the atria. This is called a heart block.

After you have caused a heart block, stimulate the ventricle electrically with a stimulator. Does the ventricle contract? What do you conclude?

EQUIPMENT

Per student

dissecting microscope
compound microscope
microscope slides
prepared slide of striated, cardiac, and smooth muscle
petri dish
fine forceps
plastic ruler

Per 4 students

solution of 0.25% ATP in 0.05-*M* KCl + 0.001-*M* $MgCl_2$
solution of 25% ATP
solution of 0.05-*M* KCl + 0.001-*M* $MgCl_2$ (above three solutions in 10-ml dropping bottles)
15% glycerol-water mixture (25 ml)
strip of glycerinated rabbit psoas muscle (soaked in 50% glycerol and water mixture at 0° for 2 weeks; changed to cold 15% glycerol and water mixture 1 hour before using) (preparation of muscle fibers described in text; see also A. Szent-Györgyi's book: *Chemistry of Muscular Contraction*, Academic Press, New York, 1951)

Per 2 students

kymograph and smoked paper
frog board
kymograph lever
ring stand
needle (small)
thread
medicine droppers
acetylcholine solution (2×10^{-4} *M*)
epinephrine solution (2×10^{-4} *M*)
atropine solution (5×10^{-4} *M*) (above three solutions in 10-ml dropping bottles)
frog ringer (50 ml, in dropping bottle)
plasticene
frogs (pith one hour before using)

XX ELECTRICAL ACTIVITY OF A SENSE ORGAN: THE LIMULUS EYE

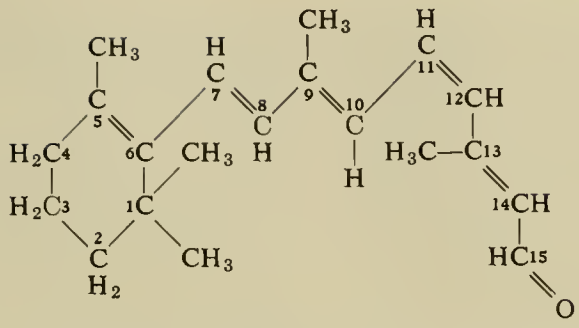
(Readings: Weisz, pp. 485–490. S. P. T., pp. 188–201. L. J. Milne, "Electrical Events in Vision," *Sci. Am.* **195**, No. 6, 113–122, Dec. 1956. W. R. Loewenstein, "Biological Transducers," *Sci. Am.* **203**, No. 2, 98–108, Aug. 1960, Reprint No. 70. W. H. Miller, F. Ratliff, and H. K. Hartline, "How Cells Receive Stimuli," *Sci. Am.* **205**, No. 3, 222–238, Sept. 1961, Reprint No. 99.)

During the past two weeks we have studied the electrical responses in peripheral nerve axons and the spontaneous activity of the heart. Today we will examine the electrical activity of a receptor and its attached nerve. The receptor we have chosen is the eye of the horseshoe crab or king crab, *Limulus polyphemus*. Though this animal is called a crab, it is not a crustacean, but an arachnid, closely related to the spiders. Many of the characteristics of its eye are shared by all types of eye, and indeed by all other types of sensory receptor.

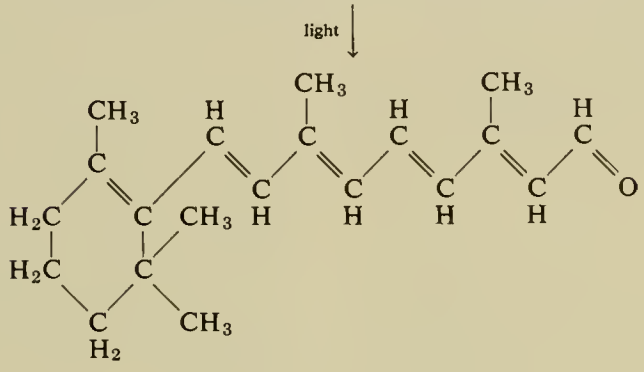
The sensory receptors are the outposts of the nervous system. Their business is to translate various types of stimuli into meaningful patterns of nerve impulses. The stimulus is always some exchange of energy or material with the environment. This may be light (photoreceptors), heat (hot and cold receptors), mechanical (touch, pressure, sound), or chemical substances (taste, smell, common chemical sense). The receptors translate all such stimuli into relatively slow, local electrical potentials, that depolarize the associated nerve fibers, causing them to fire trains of all-or-nothing impulses. These are conducted to other portions of the nervous system, and sometimes eventually out again to excite muscles and glands. The more intense the stimulus, the larger the depolarization of the receptor, and the higher the frequency and greater the number of impulses in the associated nerve.

That is, the response of the receptor cell to the external stimulus is not all or nothing, but small or large, depending upon the intensity of the stimulus; and this graded potential in the receptor is then translated into frequency and number of all-or-nothing discharges in the associated nerve fibers. Such slow receptor potentials, called in general *generator potentials*, have special names in the different receptors. In an eye such as that of Limulus they are called *retinal potentials*, and the records of them are called *electroretinograms* (ERG's).

How a receptor transduces ("leads over") stimuli of all kinds into electrical activity is not known; but in photoreceptors we do know



11-*cis* retinene, the chromophore of visual pigments



all-*trans* retinene, $C_{19}H_{27}CHO$

something of the action of light on the receptor. All photoreceptors so far examined contain light-sensitive pigments, substances which are changed on absorbing visible light so as somehow to induce a nervous excitation. Each such visual pigment is composed of a colorless protein, called an opsin, to which is attached as chromophore or color group the yellow, fat-soluble substance, retinene (vitamin A aldehyde, $C_{19}H_{27}CHO$). It is for this reason that vision depends upon vitamin A: the first symptom of vitamin A deficiency in man and other animals is the failure of vision called night-blindness.

Retinene itself is very light yellow in color. The visual pigment of the Limulus eye that is formed by the attachment of retinene to opsin is red in color. It is this red pigment that absorbs the light which is effective in vision. Molecules of retinene come in a variety of shapes, *cis-trans* isomers of one another. A special, unstable, bent and twisted shape of retinene (the 11-*cis* isomer) is the only one that can join with opsin to form a visual pigment. When a quantum of light is absorbed by the visual pigment, the effect is to straighten out the retinene to the all-*trans* isomer. Somehow, perhaps by exposing an active site on the opsin which had been covered before, this leads to the depolarization and nervous excitation. (11-*cis* retinene is both bent, as are all *cis* molecules, and twisted, owing to the —H on C_{10} running into the $—CH_3$ on C_{13}, which keeps the molecule from lying flat.)

Before a molecule that has responded in this way can participate again in excitation, the retinene must be rebent and retwisted back into the active shape that can recombine with opsin to regenerate the visual pigment. That is, the effect of light on the visual pigment is both to excite vision and to inactivate temporarily the pigment. The result is a temporary decline of visual sensitivity, called light adaptation. Then some time must elapse during which the visual pigment is regenerated, and the eye regains its maximal sensitivity. This is dark-adaptation.

You must understand that though light is needed to inactivate the pigment, it regenerates equally well in light or darkness. In a steady light the pigment is continuously inactivated and continuously regenerated, so that a balance is struck between these processes, in which some pigment is constantly present, permitting vision to continue. In the dark, only regeneration occurs, bringing the visual pigment back up to its maximum concentration and returning the eye to its maximum sensitivity.

In today's experiment you will examine the retinal generator potential (ERG), dark-adaptation as measured by the ERG, the relation of the ERG to action potentials in the optic nerve, and the patterns of nerve impulses in the optic nerve.

Equipment

The light stimulus is provided by a microscope lamp, and its intensity is controlled with neutral filters inserted in the beam. The light will be focused on the eye with a condensing lens, and the duration of the stimulus controlled by raising and lowering a piece of cardboard that shuts off the beam. Before beginning the experiment, look over this setup and try it out.

Since exposed wick electrodes will be used, the preparation must be shielded. For this it is set up inside the copper cage. Make sure that the wick electrodes are connected with the binding-post terminals on the side of the copper cage. The input cable from the preamplifier is then connected to these binding posts.

EXPERIMENTAL PROCEDURE

Note. In this first part of the experiment, use as little light as possible so as not to light-adapt your preparation strongly. The Limulus eye is not stimulated by deep red light, since its visual pigment does not absorb the long wavelengths of the spectrum. Red lamps will be available, and can be used freely without affecting the preparation.

Plan to work in groups of two to four. Place the horseshoe crab on the block of wood in the shielded cage, and fasten it down with nails through the edges of the shell. Putting nails through the shell causes no more pain than cutting your fingernails.

Identify the prominent faceted eyes, so-called compound eyes. With a fresh razor blade, gently scrape the horny surface of the eye (again a painless operation). This removes the highly water-resistant waxy substance that helps to make the eye waterpoof. Don't scrape too long or too hard; it is better to do too little than too much. Then with the tip of a sharp scalpel, dig a tiny hole through the shell directly back of the eye, just large enough to admit the tip of a wick electrode.

Set the animal in position in the shielded cage, and focus the light beam on its eye, using very dim light, and exposing it only for short intervals. The cotton wicks used as electrodes will have been soaked with sea water, so as to conduct the electric current. Place one such wick on the cornea of the eye, and insert the tip of the other through the small hole behind the eye.

Convenient settings on the recording instrument are:

Preamplifier magnification	= 1000
Indicator amplification	= 0.05 volts/division
Waveform duration	= 1000 msec (1 sec)

The trace should be steady and should not exhibit waves due to interference from the 60-cycle power lines. If you do have hash on the screen. readjust the electrodes to make a better contact, If the hash persists, you may have to scrape the surface of the eye a little more, but consult the instructor first.

Stimulate the eye with a dim, brief flash of light (through density 2.0 filter), and observe the response. How long does the response last compared with the stimulus? How does it compare in duration with a nerve action potential? Let the animal dark-adapt for a few minutes, and stimulate the eye again. If the response has grown, let the animal continue to dark-adapt until the responses have become constant. This may take up to 15 minutes or longer.

Compare the responses elicited with three different *durations* of the stimulating flash, of about $\frac{1}{2}$ sec (as fast as you can move the cardboard), 1 sec, and 3 sec. What is the relationship between the ERG and duration of stimulus at constant intensity?

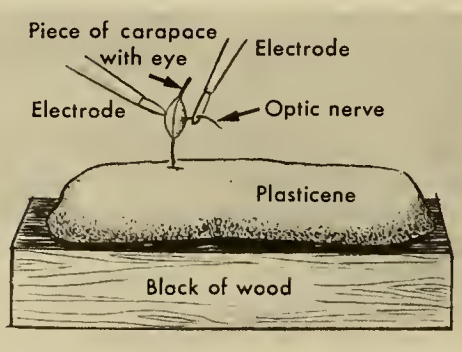
Now investigate the way in which the ERG varies with light *intensity*. The intensity is controlled with a series of neutral filters. A neutral filter is one that absorbs light more-or-less equally throughout the spectrum, and hence looks colorless (gray). Such filters are graded on a density scale, in which density equals log 1/transmission. That is, a filter that transmits 1/10 of the light has density log 10 = 1.0; a filter that transmits 1/100 of the light has density log 100 = 2.0; and so on.

Starting with the dimmest light (neutral filter, density 3.5), and a stimulus of 1 sec, measure the height of the ERG. Now, keeping the duration of the stimulus constant, progressively increase the light intensity by steps of 0.5 log unit; i.e., use progressively lighter filters in which the density falls by steps of 0.5. Make at least two measurements at each intensity that agree with each other. Wait at least one minute between exposures to allow the animal to recover. At the higher intensities you will probably have to readjust the amplification setting on the indicator to keep the response on the screen. Plot the magnitude of response (in millivolts) against the light intensity in log units. How big is the range of light intensities over which you find the response to vary? How big is it in ordinary arithmetic units? Describe the relationship between intensity of stimulus and the ERG in words, and draw what conclusions you can concerning the animal's capacity to respond to and distinguish various brightnesses of light.

Using a moderate intensity of light (density 1.0), and a 1-sec exposure, remeasure the magnitude of response to a flash. Light-adapt the animal for 5 minutes with the brightest light available, and remeasure the response at density 1.0. Let the animal remain in the dark, and periodically remeasure the response to a flash of this intensity and duration of light. Start by making a measurement every minute, and as the change slows down lengthen this interval, eventually to every 3 to 5 minutes. (Don't go on with this longer than 30 minutes.) How long does the horseshoe crab take to dark-adapt? Make a graph of the relation between the height of the ERG and time in the dark.

Remove the animal from the cage and kill it by turning it on its back and slitting it up and down the middle with a scalpel. Prepare to expose the optic nerve by first cutting a square, about 1 inch on a side, through the carapace around the unused eye of the animal, using a sharp scalpel or one-edged razor blade. Then carefully raise this piece of carapace at its upper edge, and begin to free it from the underlying tissue with the blunt end of a scalpel. Work very slowly, watching carefully for the optic nerve. It is a *very fine*, glassy structure that runs forward from the eye. If you have trouble, call an instructor.

When you find the nerve, free it from the bulk of the surrounding connective tissue and tie a suture around its distal end. Now remove the square of carapace containing the eye with its attached nerve from the animal, and continue to clean away the connective tissue from its back. Go as far with this as you can, but be very careful not to damage the eye itself. The cleaner the nerve, the better your experiment will go. Keep the nerve moist with sea water.



Mount the eye upside down, as shown in the diagram, on a block of plasticene. Position the block of plasticene in the shielded cage, and re-

focus the light on the eye. Touch one wick electrode to the front of the eye, and sling the optic nerve over the other wick electrode. Be sure that the wick touches nothing but the nerve. On stimulating the eye, you should see small nerve impulses superimposed on the ERG. Remember that the eye has probably been light-adapted during your manipulations, so if the responses seem small, wait a few minutes for them to grow larger. (Getting good reponses from a preparation like this may take some fussing. If your responses are small, or none is visible, try readjusting the nerve on the electrode. It is usually advantageous to have the electrode close to, though not touching, the eye. If the nerve is too wet, the responses may be shorted out by the sea water; and the nerve should be dried with a bit of cotton. On the other hand, if the nerve is too dry, it will not make suitable contact, and should be moistened. So if your responses are not ideal, keep fiddling and don't get discouraged. Your instructor may also have suggestions.)

Examine the relationship between the intensity of the light, the height of the ERG, and the relative number of impulses in the optic nerve. Examine also the responses to short and long flashes at one intensity. Describe your observations and draw conclusions.

If you wish to study the nerve impulses alone, you can filter out the ERG by turning the low-frequency dial on the amplifier to the 80-cycle setting. This makes the amplifier unresponsive to signals that have a time course longer than 1/80 sec. Examine the effect of a long flash of light on the train of impulses. What changes in frequency of impulses do you see? At what point, relative to the onset of stimulus and the shape of the ERG, does the nerve response reach the highest frequency? Does the response stop completely after the stimulus has been on for a time? What would you conclude of the animal's sensations?

It seems to be a general rule that receptors respond most strongly to change, rather than to steady stimulation. Demonstrate this for yourself by flickering the light to the Limulus eye by rapidly moving the cardboard back and forth through the light beam.

It is possible to separate out single fibers from the optic nerve of Limulus. If you have time at the end of the experiment, try teasing out small bundles of fibers with glass needles and fine forceps. Move both electrodes to the back of the eye, and sling one such nerve bundle across both wicks. If you are lucky, you may be able to separate out a bundle that contains only one or a few active fibers. This is not an easy thing to do, and several tries may be necessary.

EQUIPMENT

Per 2–4 students

electronic recording equipment
copper cage (2 ft square)
pair of wick electrodes (see notes on electronic equipment)
wooden block, 3″ × 3″
2–3 nails
3 neutral density filters (0.5, 1.0, 2.0) (partly exposed films will do)
condensing lens
microscope lamp, wired for d-c
piece of cardboard, 8″ × 10″
2 blocks of plasticene, 2″ × 2″
2 glass needles
flashlight with red cellophane filter
limulus, 2″ to 4″ across carapace
thread
razor blades

XXI PLANT GROWTH AND TROPISMS; CARBON DIOXIDE FIXATION AND TRANSLOCATION OF PLANT SUBSTANCES

(Readings: Weisz, pp. 253–263. S.P.T., pp. 183–185; 57–63. Villee, pp. 107–113; 126–127. Review Exercise X on "Photosynthesis." G. Wald, "Life and Light," *Sci. Am.* **201,** No. 4, 92–108, Sept. 1959, Reprint No. 61.)

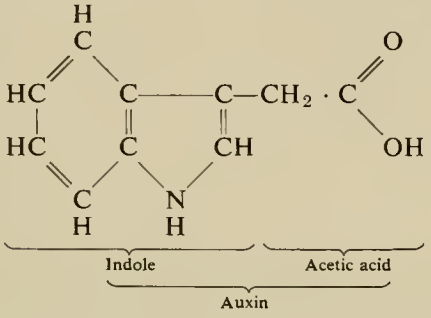
All organisms respond to stimuli, though not all of them with as swift integration and motions as provided by the neuromuscular systems of higher animals. Plants, for example, from unicellular molds to flowering plants, respond to a variety of stimuli with appropriate motions.

When we plant seeds in the ground, for example, we pay no attention to how they are oriented, yet the stems always grow upward and the roots downward. Similarly, in any situation in which light comes regularly from one side, plants tend to bend toward the light.

These responses are obviously highly advantageous, directing the organs of the plant where they can do the most good. Such directed motions in response to directional stimuli are called *tropisms.* (If the entire organism, rather than one of its parts, moves toward or away from the stimulus, this is sometimes called a *taxis.*) In the case of growing upward or downward, the force is gravity, and the direction is the center of the earth. We speak of such responses as *geotropisms,* and distinguish the directions toward and away from the center of the earth as positive and negative. So one describes the growing downward of roots as positive geotropism, the growing upward of shoots as negative geotropism. Similarly, bending toward the light is positive phototropism, whereas bending away from the light would have been called negative phototropism.

Since they lack contractile tissues, plants perform these motions by differential growth. Light, for example, inhibits the axial growth of shoots. Hence the side toward the light grows more slowly than the shaded side, with the result that the shoot bends toward the light. Some of the lower invertebrates that are attached as are plants exhibit similar tropisms. The hydroid *Eudendrium,* for example, a coelenterate, bends toward light by differential growth, just as does a plant.

As you know, plants of all sizes and ages always retain meristematic tissue that is capable of new growth. In a young shoot, growth in length is confined to a rather narrow zone toward the tip. This growth is controlled by a hormone called *auxin*. The most prominent auxin is indole-3-acetic acid (IAA), which has the following formula:



Auxin acts by promoting cell elongation, rather than cell division. It is synthesized in the tip of the shoot, though small amounts of auxin are also produced in roots, leaves, and fruit. The auxins are distributed throughout the plant from the apical buds via the phloem. The highest concentration of auxin is found nearest the apical bud, and the concentration falls off rapidly toward the basal portions of the plant. Auxin is inactivated or destroyed during growth, and must be continuously supplied from the apical bud. Within the range of low auxin concentrations, if one portion of a plant has more auxin than another, it grows faster. The differential distribution of auxin accounts for much of the differential growth, and hence the tropisms of plants.

Among today's experiments, you will have the opportunity to examine the effects of auxin on growth, the responses of plants to light and gravity, and other aspects of the physiology of plant growth. We shall use for these experiments the classic oat shoot (*Avena*). The young shoot consists of a colorless tubular sheath, the coleoptile, which surrounds the yellow or green primary leaf. It is the coleoptile that is principally responsible for the bending reactions. We shall also take the opportunity to examine under the microscope the tissues of a higher plant concerned with growth and translocation.

One of the most useful techniques developed for investigating cellular metabolism depends upon isotope-labeled molecules. Such molecules have exactly the same chemical properties as those lacking the label, and can be used to follow the pathways and ultimate fates of metabolites in the organism. Today, we shall offer CO_2 labeled with the radioactive isotope of carbon, C^{14} (therefore $C^{14}O_2$), to a bean leaf, and investigate its uptake in light and darkness, and the subsequent translocation of the carbon compounds newly synthesized from it.

Radioactive compounds emit radiations that, like light, affect a photographic film, producing a latent image which darkens on development. We will measure both the uptake and distribution of the radioactive carbon in the leaf by exposing a film to it.

Carbon-14 is a relatively stable radioisotope that emits β-rays (electrons). This is not a very penetrating radiation; one thickness of paper can usually block it. For this reason C^{14} compounds are relatively safe to use; yet *take care* with them. Be careful not to spill any radioactive materials. Also place any contaminated materials as soon as you are through with them into the special containers which are provided. Wash your hands thoroughly before leaving the laboratory.

PLANT GROWTH AND TROPISMS

First test the effects of auxin on the growth of the stem. You will be supplied with 4 oat seedlings that are 3 days old (the first leaf should not as yet have pushed through the coleoptile). With a razor blade cut off the terminal 3 mm of each tip, and discard it (why?). Then, using a sharp razor blade, cut a segment exactly 10 mm long from each plant. Place 2 such segments in each of 2 small petri dishes. Fill one dish with a 2% sucrose solution, the other with 2% sucrose containing 2 mg per liter of indole acetic acid, brought to a slightly acid pH with KH_2PO_4. Place the dishes in the dark (your desk drawer). After at least 2 hours have passed, measure the length of the seedlings to the nearest quarter

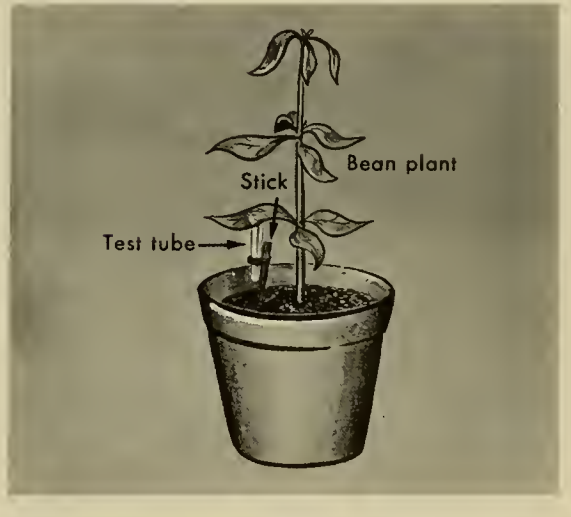
millimeter, under the dissecting microscope. If you leave the experiment until the following morning, your results will be much plainer. Calculate the percentage increase of length per hour in each of the solutions.

Next, working in groups of four or eight, test the effects of light and gravity on *Avena* seedlings. Each group should obtain a dish containing young seedlings. Weed out any seedlings that are not straight. Place the dish under the wooden box that is provided, at the end away from the aperture that holds a light filter. At the various tables in the room, the boxes contain different light filters, red, yellow, blue, and green. Move a microscope lamp close to the aperture, so that light penetrating the filter reaches the plants. Irradiate the plants in this way for 90 minutes. Then note whether or not they have bent toward the light, what proportions have responded, and about what angle the tip of the plant assumes with the vertical. To make this measurement more quantitative, lay the plant on a piece of graph paper and trace the bend. You should get a sufficiently accurate measurement of the degree of bending to compare with your neighbors' results.

By comparing your results with those obtained at other tables, grade the effectiveness of the different colored lights in stimulating bending. Draw a graph of this effectiveness against wavelength in the spectrum (representative wavelengths: violet, 410 mμ; blue, 470 mμ; green, 520 mμ; yellow, 580 mμ; orange, 600 mμ; red, 650 mμ). Such a graph, when corrected for the energy content of the various colored lights, is called an *action spectrum*.

Phototropic bending in plants, like vision in animals, is mediated by light-sensitive pigments. This is necessarily true; for light in order to have any effect, chemical or physical, must be absorbed; and substances that absorb visible light are pigments. The effectiveness of the various wavelengths of light in stimulating vision or phototropism depends in the first instance on the capacity of the photoreceptor pigments to absorb those wavelengths. Hence an action spectrum tells us not only the region of the spectrum most effective in stimulating the response, but by the same token the region of the spectrum most strongly absorbed by the photoreceptor pigment. This tells us the color of the pigment, and sometimes provides a clue to its chemical nature.

Of the various pigments present in *Avena* seedlings, the chlorophylls *a* and *b* absorb light in the blue and red, and hence are green in color; whereas the carotenoids, xanthophyll and carotene, as also riboflavin, absorb light only in the blue, and hence look yellow. Judging by your observations, which of these pigments might possibly mediate the phototropic response?



CO_2 FIXATION AND TRANSLOCATION

In this experiment, work in pairs. Obtain a bean plant and a small test tube which contains 1 to 2 μgm of radioactive barium carbonate from your instructor. (*Be careful* not to spill any of the carbonate; if you do, tell your instructor so that he can get rid of it.) Tape the test tube to one of the sticks provided, and place the stick upright in the earth surrounding your bean plant, directly underneath one of the bean leaves. Adjust the height of the stick so that the leaf rests firmly against the mouth of the test tube, as shown in the diagram. Gently push the

leaf aside, and ring the top of the test tube with vaseline. Carefully introduce 2 drops of H_2SO_4 into the bottom of the test tube with the capillary tube that is provided. (Your instructor will demonstrate this for you.) Make sure the acid reaches the bottom of the tube, and that there is not an air bubble holding back the second drop of acid. If there is, hit the tube sharply with a snap of your finger, until the acid falls to the bottom of the tube. Also, be careful not to allow any acid to wet the top of the test tube.

As soon as the acid is in the test tube, quickly replace the leaf, very gently pushing it down until coming in contact with the vaseline, it is sealed over the mouth of the test tube. The leaf should now be left undisturbed for 10 minutes, while the plant is brightly illuminated with the lamp that is provided.

You should be able to see small bubbles of $C^{14}O_2$ rising through the acid. This is formed by the reaction:

$$BaC^{14}O_3 + H_2SO_4 \longrightarrow BaSO_4 + H_2O + C^{14}O_2.$$

After exactly 10 minutes, cut off the leaf at the base of its stem with a pair of scissors. Carefully wipe off the vaseline, and lay the leaf flat within a folded piece of paper that is marked with your name and the letter "A." Place the paper in the refrigerator.

Now obtain another test tube with radioactive carbonate, and repeat the above experiment on another leaf, except that immediately after the leaf has been placed over the test tube generating $C^{14}O_2$, cover the whole plant with the black hood that is provided. Again, expose the plant for exactly 10 minutes.

At this point, remove the black hood and as quickly as possible place the test tube generating $C^{14}O_2$ under a third leaf. Cut off the second leaf as you did the first, remove the excess vaseline, lay it within a folded piece of paper marked with your name and "B," and also place it in the refrigerator.

To prepare the third leaf, again brightly illuminate the plant, and leave it undisturbed for 25 to 30 minutes. Now remove the leaf, and prepare as before, marked with your name and "C."

After the third leaf has been in the refrigerator for at least 10 minutes, remove all three leaves from the refrigerator and take them into a room which has been outfitted as a darkroom, illuminated only with dim red light.* There take two pieces of x-ray film and cut off one corner of each piece to identify an end. (Try to perform all manipulations with the x-ray film in the dark, or as nearly so as you can manage. The film rapidly fogs when exposed to light, even to red light.) Place the three leaves in the order A, B, C from top to bottom on one piece of film, with the cut corner at the top. Cover this with the second piece of film, also with the cut corner at the top, so that the leaves lie between the two emulsions. Sandwich the films between two pieces of cardboard, holding everything together with rubber bands around each end. Place the sandwich in a black envelope, seal with rubber bands, mark with your name, and put the package into the freezer of a refrigerator.

Sometime during the next laboratory period develop your film in the dark room. Again try to work in as little light as possible. Remove the film from the sandwich, and throw the radioactive leaves in the can which is provided. Attach a clothespin to one side of each piece of film and immerse both films in developer for 3 minutes, then rinse in water, and immerse both in fixer for 5 minutes. Then wash the films in running water for at least 10 minutes before looking at them.

(*Caution:* Photographic developer stains, and fixer eats at clothing. Keep both from dripping around; and be particularly careful to keep any trace of fixer out of the developer.)

Wherever a β-particle from the radioactive carbon hits the film emulsion, a silver ion is reduced to metallic silver, which in the developer catalyses the reduction of a whole grain of the emulsion, resulting in a black spot. Compare the patterns and intensity of radioactivity

*If a Geiger counter is available, count the radioactivity incorporated into each leaf at this point and record the results in your notes.

in the three leaves as displayed by your film. What do you conclude about the effects of light on the incorporation of CO_2? What evidence do you observe of the translocation of recently synthesized organic molecules?

In the intervals of waiting for things to happen, study the prepared slides of the apex of the flowering plant *Coleus*. The small, darkly stained cells that form a small mound at the apex of the stem represent the apical meristem. Remember that these cells are responsible for the further growth of all the remaining plant structures that are above ground. Note the young leaves developing around the apex. Careful observation will reveal much of the differentiation of the tissues that compose the lateral stems and leaves. Note the area that will become vascular tissue. Follow bundles of vascular tissue back to the apex from the largest leaves and the base of the stem.

Tissue differentiation is much easier to study in plants than animals, because all stages of differentiation appear in a linear sequence starting at the apical meristem and working toward whatever type of tissue interests you.

EQUIPMENT

Per student

8 *Avena* seedlings, grown 3–5 days
2 petri dishes
2% sucrose solution (5 ml)
2% sucrose solution with 2 mg/l indole acetic acid, brought to pH 6.5 with KH_2PO_4 (5 ml)
razor blade
graph paper
0.5-ml centrifuge tube containing $BaCo_3$
wooden sticks (12″ long)
piece of 5″ × 7″ (no screen) x-ray film
piece of 5″ × 7″ cardboard
2 rubber bands
black paper envelope (8″ × 10″)
prepared slide of apex of *coleus*

Per 2 students

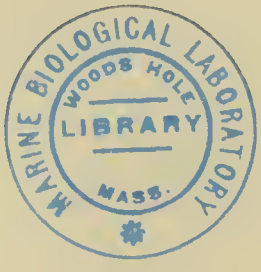
bean plant (2–4 weeks old)
black wooden box with colored light (red, green, blue, and yellow) filter

Per 8 students

roll of Scotch tape
H_2SO_4, 1 *M* (5 ml) and capillary pipet
jar of vaseline

Per laboratory

space outfitted as a darkroom
developer and fixer for processing x-ray film



XXII INTRODUCTION TO THE GENETICS OF MAN AND THE FRUIT FLY; REGENERATION OF PLANARIA

(Readings: C. M. Williams, "The Metamorphosis of Insects," *Sci. Am.* **182**, No. 4, 24–28, April 1950, Reprint No. 49. Weisz, Chapter 27. S.P.T., pp. 159–161; 240. Villee, pp. 496–508. R. Buchsbaum, *Animals Without Backbones*, Univ. of Chicago Press, rev. ed., 1948, Chapters 10 and 12.)

In this laboratory section, beyond considering a few simple examples of human genetics, we will begin a four-week program of genetics experiments on the fruit fly, *Drosophila*. While these experiments develop, we shall have ample time to do other things. During the present period you will begin experimenting with the regeneration of a planarian. There will also be slides on display demonstrating meiosis and mitosis in a variety of animals and plants; and also stained preparations of the giant chromosomes of the salivary glands of *Drosophila* larvae.

ASPECTS OF HUMAN GENETICS

PTC tasting

The substance phenylthiocarbamide (PTC; phenylthiourea) tastes very bitter to some persons ("tasters") but is tasteless to others ("non-tasters"). The ability to taste it is inherited as a dominant characteristic. About 70% of the American population taste PTC, the other 30%, who are homozygous for the recessive allele, do not taste it.

Pieces of paper which have been impregnated with PTC will be provided. Hold a piece in your mouth for about 30 seconds to determine whether or not you are a taster. How does the class come out as a whole?

Excretion of methyl mercaptan

Asparagus contains the organic sulfur compound dimethylthetin ($(CH_3)_2S^+—CH_2COOH$). About 60% of the American population possess an enzyme which catalyzes the conversion of dimethylthetin to methyl mercaptan (CH_3SH). It is the latter substance that gives urine its characteristic odor after asparagus is eaten. The presence of the enzyme is a dominant trait.

Sex-linked genes in man

The most common sex-linked human trait is red-green color blindness. This occurs in about 8% of the male and 0.5% of the female population. The recessive gene responsible for color blindness is in the X chromosome, and since men have only one X chromosome, while women have two, a father transmits his X chromosomes to all his daughters but never to his sons, whereas a mother gives one X chromosome to each of her children regardless of sex. It follows that the sons of a color-blind mother are all color blind, but daughters have normal vision if the father has normal vision. The daughters, however, carry the color-blindness trait; if married to men with normal vision, their daughters are normal, but half their sons are color blind. How is a color-blind woman produced?

Hemophilia, the failure of the blood to clot, is another sex-linked recessive trait, also therefore almost entirely restricted to males. One of the troubles with European royalty is that Queen Victoria, a carrier of the hemophilia gene, tended to have royal descendants who bled for the wrong reasons.

Other human genetic traits

You have already typed your own blood. (See page 327 of S. P. T., or pp. 471–472 in Villee, for a description of genetic aspects of blood types.)

You may be interested in the following examples of other human Mendelian traits:

Blood types Rh^+, Rh^-
Tongue rolling
Tongue folding
Widow's peak
Dimpled cheeks
Mongolian eyefold
Hyperextension of distal thumb joint
Albinism
Straight hair, curly hair
Mid-digital hair on fingers
Far-sightedness
Near-sightedness
Astigmatism
Attached or free ear lobes
Full lips, thin lips
Freckles

DROSOPHILA GENETICS

The common fruit fly, *Drosophila melanogaster* (i.e., "black-belly"), has been highly important in genetics since introduced half a century ago by T. H. Morgan. Its short generation time, ease of handling, large number of offspring, and convenient size all tended to make this the most widely used organism in genetics. Only lately has it been superseded by microorganisms, which offer still further conveniences and potentialities for experiment, once one has learned to handle them.

A further advantage of *Drosophila* is that it possesses as the diploid number only four pairs of easily identified chromosomes. Also the salivary glands of the larvae contain giant chromosomes, the structures of which have provided important anatomical correlations with genetic linkage maps, and which have furthered the analysis of chromosome functions and rearrangements.

Overall plan of the experiment

We have planned an experiment that demonstrates Mendel's laws of segregation and independent assortment. It involves two recessive mutants, the genes for which are located in separate chromosomes: dumpy (*dp*) and ebony (*e*). Flies homozygous for dumpy have truncated wings, only about two-thirds as large as wild type. Those homozygoous for ebony have shiny black bodies, much darker than wild type.

A week before this laboratory session initial crosses were made between males and virgin females, the flies of one sex taken from a stock homozygous for dumpy body, the other from one homozygous for ebony. The parent flies remain in the vials that you have been given, and will shortly be removed. The eggs already laid by these females will hatch to form the F_1 generation with which the experiment will be continued.

The schedule for the entire experiment is as follows:

This week: Remove the parent flies, following the directions below.

Week 2: Cross the F_1 flies, and record their phenotypes.

Week 3: Remove the F_1 parents.

Week 4: Score the results of this cross and of a more complex cross which will be given you.

Each week you will find detailed instructions for proceeding with the experiment.

This week's work

The main job this week, apart from removing the adults from your vials, is to get to know the flies and learn to handle them.

Begin by removing the adults from the vials, etherizing them as described below. Have the vials ready beforehand to be stored for incubation. Be sure each vial is labeled with your name and a description of the cross and the date it was made. Each mutant gene has a special symbol, *dp* for dumpy, *e* for ebony, and + for wild type. We will use diploid formulas that represent the somatic cells of the parents, rather than haploid formulas that would represent mature germ cells. The formula for the female should be written on the left, followed by × and the formula for the male. The pair of gene symbols for each chromosome pair is written one above the other, like a fraction; so for example, homozygous dumpy is represented by $\frac{dp}{dp}$. The initial cross, therefore, in which homozygous dumpy females were mated with homozygous ebony males, can be written $\frac{dp}{dp} \times \frac{e}{e}$.

As soon as the adults have been taken out of the vials, return the vials to the boxes so that they can be incubated until next week.

The etherized adults should be examined carefully under the dissecting microscope. You should be able to distinguish males from females and the mutant types from wild-type flies, several of which will be provided for comparison. You will find descriptions to guide you below and in your reading.

Examine also *Drosophila* eggs, larvae, and pupae under the dissecting microscope. These stages will be given you. Do not take any eggs from your experiment.

In any free time, examine the prepared slides which are set up under the demonstration microscopes.

Life cycle

At 25°C the entire life cycle of *Drosophila* is usually completed within 10 days. It includes four stages: egg, larva, pupa, and imago (adult), as in all Diptera (true flies). The eggs, about 0.5 mm in length, are sausage-shaped white structures bearing a pair of filaments at the end, which help to keep them from sinking into the soft food on which the eggs are always laid. The larvae are little white maggots which burrow in the food at the bottom of the vials. *Drosophila* larvae undergo two molts after emerging from the egg; the larval period thus consists of three stages (instars). Larvae may be up to 4.5 mm long in the final stage; it is from them that the giant salivary gland chromosomes are obtained. At the end of the third instar, the larvae crawl up to a dry spot on the wall of the container, where they pupate in small dark cocoons. Pupation lasts about four days at 25°C, after which the adult fruit fly emerges. The adult is at first light in color, and its wings are crumpled; but within a few hours the wings expand and the adult takes on its familiar appearance.

Determining the sex of adults

Males can be told from females with the naked eye, using several different criteria. Though the external genitalia are more complex in males, this difference is difficult to see. The abdomen of the female is long and pointed at the end, whereas that of the male is considerably shorter and somewhat stubby. Furthermore, the entire rear portion of the male abdomen is black, whereas in the female dark and light bands alternate to the tip. One of the

most helpful signs of maleness is the possession of "sex combs," consisting of a series of about 10 stout, black bristles on the basal (upper) tarsal joint of the first legs; these can be seen with the naked eye.

Your initial matings were made with virgin females. These are obtained by emptying all the adults out of an active culture of *Drosophila*. One or two hours later, one finds a few new adults that have emerged in the interim, and have surely not yet mated. If one now segregates such virgin females, they can be kept until wanted for mating. The males of course don't require such precautions; they can be taken from the culture at any time.

Drosophila culture

Drosophila can be raised in the laboratory in 3-inch glass vials closed with cotton plugs and held at a constant temperature of 25°C. The food consists of a cooked-up mixture of corn meal, agar, molasses, water, and a mold-preventive. The hot food is poured into vials, and allowed to cool, the mass being stiffened by the setting of the agar into a gel as it cools. A thin suspension of yeast sown on the surface of the food grows rapidly on this medium, providing food for the flies. If the food is too soft or the vial too wet, the adult flies readily stick to the walls or drown. Precautions must be taken to prevent these things from happening. It is important also that flies not be allowed to escape into the laboratory, since adventitious matings could invalidate your results. Containers are provided for the disposal of used vials, and flies with which you have finished, first killed by overetherizing, should be placed in the "morgues" (jars containing kerosene oil).

Handling *Drosophila*

Flies are anesthetized with ether to keep them quiet during examination or transfer. Care must be taken not to overetherize them; there is only a narrow gap between anesthetizing and killing them with ether. Flies killed in this way can be recognized, since their wings are drawn up away from the abdomen, the proboscis is everted, the legs are stiffly extended and bunched together, and the body is curled and has stopped twitching.

There are two types of etherizer available. To use the plastic Burco model, put not more than two drops of ether into the chamber through the spout. The ether should last an hour or more. Shake the flies to the bottom of your vial; remove the vial top and place the funnel top over the open vial. Now invert the vial and etherizer together and tap gently to shake flies into the chamber. Immediately after the last fly becomes still, remove the cap at the bottom of the chamber and pour the flies out.

The other type of etherizer is made of a glass bottle with a tightly fitting cork holding a piece of cotton. To use it, drop a few drops of ether on the cotton, quickly shake the flies from the culture bottle into the etherizer bottle, and quickly close it with the cork, with its ether-wetted cotton inside. (Be sure that the cotton on your etherizer plug is just moistened, not soaked with ether. Any liquid ether that touches the flies is instantly fatal.) Some practice may be needed to do this smoothly. It is helpful first to tap the culture vial sharply against the palm of the hand, so as to shake the flies away from the cotton plug, yet not so violently that they become stuck in the food. Immediately pull out the plug, and set the mouth of the culture vial into the mouth of the etherizer bottle, holding the latter down. Tapping lightly on the upturned bottom of the culture vial, and holding the etherizer bottle toward the light, help to get the flies into the etherizer bottle. Don't tap so hard as to knock pieces of food in on top of them.

Caution: Since ether is dangerously explosive, there must be no flames or lighted cigarettes in the room.

At most 10 seconds after the flies in the etherizer have stopped moving, empty them out for examination. If the anesthetization wears off before you have finished examining them, they can be re-etherized. A re-etherizer is made from one section of a petri dish, with a piece

of absorbent cotton or paper taped on the inside. A drop or two of ether is put on the cotton or paper, and the dish is placed over the flies for a few seconds. Alternatively, the flies can be covered with the open chamber of the plastic etherizer. Flies are more easily killed by a second exposure to ether than by the first, so be particularly careful not to overdo it.

The anesthetized flies should be dumped out of the etherizer onto a white paper card and examined under the dissecting microscope; use whatever magnifications are convenient. The flies are moved around on the card with a camel's hair brush or a dissecting needle. When dividing the sexes, it is convenient to line up all the flies at the center of the card and then to run down the line pushing males to one side and females to the other.

REGENERATION OF PLANARIA

Planarians have a remarkable capacity to regenerate parts of their bodies which have been removed. Regeneration occurs in all animals, yet to different degrees, tending to diminish as one ascends the evolutionary scale, until in mammals it is restricted to wound-healing.

Planarians are members of the phylum *Platyhelminthes*, the flatworms. (For their systematic position, see Weisz, pp. 731–732; S. P. T., pp. 528–531; Villee, pp. 201–203.) They are small animals, less than an inch long, and have a primitive brain, eyes, digestive organs, muscles, and an excretory system. They reproduce either sexually or by fragmentation.

Our planarian is *Dugesia dorotocephala*, a relatively large species that is uniformly darkly pigmented. It is found in the middle-western states in wells or spring-fed streams. In the laboratory, planarians are kept in spring water or dechlorinated water, and are fed occasionally on bits of beef liver. During the course of regeneration, however, they should be starved.

Each student will be given two or three animals. It will be up to you to design your own experiments to demonstrate regeneration. This laboratory guide is only to suggest possibilities. Everyone should read the chapters in Buchsbaum's book before coming to the laboratory in order to see the variety of simple experiments that can be done. After the initial operations have been performed, the animals must be disturbed as little as possible. If you would rather carry out this experiment at home, perform the surgery there also.

Before operating on the animals, you might determine their sensitivity to light. They have well-defined eyes, which can discriminate brightnesses and the direction from which light comes, but which probably do not resolve images. Use the lamp from the dissecting microscope as a light source. Simpson, Pittendrigh, and Tiffany (p. 240) and Buchsbaum (pp. 118–120) describe several experiments on the behavior of these animals, even one experiment that suggests learning. You might want to repeat these or to devise experiments of your own.

Planarians are best observed under low powers of the dissecting microscope, in either a small petri dish or on a slide. Be sure to use a preparation of bicarbonate-versene-tap water (BVT) and never the untreated tap water, which may kill them. They are best transferred from one container to another with a small camel's hair brush, or with a bit of tissue paper grasped in forceps so as to serve as a brush. To make a cut, wait until the animal has flattened out, and then make a quick slash, perpendicular to the plane of its body, with a clean, sharp razor blade. After the operation, transfer the animal or its parts to the containers and label them carefully. Keep them cool, though not cold, and in little or no light. The water should be replaced two or three times a week with fresh BVT, and dead animals must be removed at once. Do not feed them during the month or so it will take to complete regeneration. The animals should be left in the laboratory and disturbed as little as possible. They are quite fragile after the operation and will disintegrate if shaken.

Experiments of this kind have disclosed a number of principles which govern regenera-

tion. Two of these are: (1) The pieces of the animal retain the polarity they had in the whole animal; a new head grows from the end originally nearest the head, and a new tail from the end originally nearest the tail. (2) Pieces cut from near the head regenerate better than those from near the tail. Your own experiments can demonstrate both points. It has been suggested that differences in the rate of metabolism, graded downward from the anterior end, may explain the polar nature of regeneration. The regenerating parts at first lack the pigmentation of the original tissue and are thus easily recognized.

Planarian monsters possessing two heads or two tails can be made by slicing the animal parallel to the long axis of the body, the cut extending about a third of the body length, either through the head or through the tail. Since there is a great tendency for the divided parts to rejoin and heal together, the slit should be reopened every day if necessary.

EQUIPMENT*

Per laboratory

prepared slides demonstrating meiosis and mitosis

stained preparations of salivary glands of *Drosophila* larvae

ether in dropping bottles

PTC paper

white cards

dissecting microscope for each student

etherizers† and re-etherizers

"morgues" (jars containing kerosene oil)

razor blades

camel's hair brushes

Dugesia dorotocephala (2 or 3 per student)

petri dishes (2 or 3 per student)

solution of bicarbonate, versene, and tap water (BVT), prepared as follows:

(1) $NaHCO_3$ (2 gm/100 ml); sodium (di)ethylenediamine tetracetate (sodium versenate) 1 gm/100 ml

(2) $CaCl_2$ (1.5 gm/100 ml)

To prepare 1 liter of BVT, put 5.0 ml of solution (1) and 5.0 ml of solution (2) into some *hot* tap water, and then make up to 1 liter with hot tap water. (Using hot water gets rid of the chlorine faster.) The solution can be used after standing overnight.

*Detailed information about designing experiments with *Drosophila* and obtaining stocks can be found in the *Drosophila Guide*, by M. Demerec and B. P. Kaufmann, which can be obtained for 25¢ from the Carnegie Institution, 1530 P St. N. W., Washington 5, D. C.

†Etherizers can be made from small, wide-mouthed bottles to the corks of which have been tacked bits of cotton. Polyethylene anesthetizers can be bought from Burdick Drosophila Supply Co., 250 Lincoln Street, West Lafayette, Ind.

XXIII FERTILIZATION AND EARLY DEVELOPMENT; CONTINUATION OF THE GENETICS EXPERIMENT

(Readings: Weisz, pp. 532–536; 594–601. S.P.T., pp. 335–340. Villee, pp. 420–421; 430–432.)

During fertilization, a haploid sperm nucleus fuses with a haploid egg nucleus to form the diploid nucleus of a new cell, which by repeated mitoses and differentiation develops into an adult organism. All the somatic cells of the adult organism, including the precursors of the mature germ cells (spermatocytes and oocytes) have the double chromosome number ($2n$). As part of the process of maturation of the germ cells, this is halved in the reduction division of meiosis. The sperms that engage in fertilization are wholly mature and haploid. In most animals, however, the egg does not complete its maturation until after the sperm head has entered it. The egg nucleus then completes its meiosis, throwing off the supernumerary nuclei in one or two polar bodies and achieving the haploid condition just before fusing with the sperm nucleus. In many coelenterates and echinoderms, the egg has finished its maturation divisions before the sperm enters, so that fusion of nuclei and cleavage can proceed immediately.

THE SEA URCHIN

During this laboratory period we shall observe fertilization and the first stages of development in an echinoderm, the sea urchin. The sea urchin can be induced to shed its eggs and sperm by passing a weak electric current through it or by injecting a small quantity of potassium chloride solution; or the ovaries and testes can be removed by dissection. It has been estimated that one sea urchin contains about 10^{11} sperm, or about 8 million eggs.

Procedure

Obtain a petri dish containing a suspension of eggs in sea water, and examine them under the dissecting microscope at convenient magnifications. Note the thick jelly coat that holds the eggs apart. Add one drop of dilute sperm suspension to the eggs, swirl gently to mix, and record the time and room temperature.

The schedule of sea urchin development varies slightly in different batches of eggs and varies

greatly with the temperature. An approximate schedule of development at 23°C for an East Coast sea urchin, *Arbacia punctulata*, is as follows:

Sperm comes into contact with egg	0 min
Completion of fertilization membrane	2
Union of pronuclei	8
Completion of hyaline layer	20
Streak stage	20–35
Nuclear membrane breaks	35
Prophase	35
Metaphase	40
Anaphase	42
Telophase	45
1st cleavage	50
2nd cleavage	78
3rd cleavage	103
4th cleavage	130
5th cleavage	157
Blastula (about 1000 cells)	7–8 hours
Gastrula	12–15 hours
Skeleton begins	19 hours
Pluteus (larva)	1 day

Other species of sea urchin have different schedules of development, usually slower.

After penetrating the jelly coat, a spermatozoön touches the surface of the egg. At this point an entrance or fertilization cone forms, within about 20 seconds, which engulfs the sperm head. The cone is very difficult to see, so don't be disappointed if you miss it. A fertilization membrane also begins immediately to form around the egg, and to lift off, leaving a space between it and the egg surface. This takes about 2 minutes. The changes that take place in the egg during the next 40 minutes or so are difficult to see *in vivo*, but slides are available showing sections of eggs in various stages of mitosis.

Depending upon the species and the temperature, about 45 to 90 minutes after fertilization, sea urchin eggs begin to undergo their first cleavage. Prepare a sample containing 30 to 50 eggs, and record the time at which the first eggs have cleaved, and then at intervals of 2–3 minutes, record how many eggs have cleaved until all that are going to have done so. Draw a graph showing the percentage of cells that have undergone first cleavage (ordinate) against time in minutes (abscissa). Draw another graph showing the percentage of cells that have cleaved per one- or two-minute interval, i.e., the rate of cleavage (ordinate) against time in minutes (abscissa).

The latter curve usually has the typical bell-shaped form of a "population curve," the distribution of any measured property in a population of independent individuals. The former curve (usually S-shaped or sigmoid) is the typical summed-over or integral form of a population curve. If in this class, for example, you measured everyone's height, and then plotted two curves—one of the number of persons in each height range (66–68 inches, 68–70 inches, and so on) as ordinate against the height as abscissa, the other curve recording the total number of persons under each height as ordinate, against the height as abscissa—you would probably obtain a similar pair of curves. (To say this in the language of calculus: the bell-shaped distribution curve is the differential form; the sigmoid curve is its integral.)

In order to determine the schedule of development through the first four cleavages, you will want to examine fertilized eggs during the first five or six hours after fertilization. Your instructor will provide you with two batches of eggs, fertilized 3 hours and 1.25 hours before the laboratory period begins. With these two batches and the eggs you have fertilized at the beginning of the period, you will have samples of fertilized eggs at all stages during the first six hours of development. Working in pairs, set up a sampling schedule so that you can follow the progress of development at half-hour intervals, using the time of fertilization of the three batches as starting times. Thus, using the egg fertilized 3 hours before the laboratory session began, you may get time intervals up to 6 hours after fertilization. A sample should be taken by placing a few drops of eggs selected randomly

from the large batch onto a depression slide. Examine under low power and count 30 to 50 eggs, classifying them according to cleavage stage (the larger your sample, the more reliable your results will be). For each succeeding count, withdraw a fresh sample from the appropriate batch of eggs. Tabulate your results when you finish according to *time* (time from fertilization to sampling) and cleavage stage (expressed as per cent of total sample counted). Make a graph showing the percentage of eggs uncleaved and in each stage of cleavage as ordinate, against the time as abscissa (use different colors for the different cleavage stages). In such a graph, the uncleaved eggs should form an S-shaped curve, the various cleavage stages bell-shaped curves. The peak of each of the latter curves represents the characteristic time for that stage of development. With these characteristic times, and noting the temperature, prepare a schedule of development for the species you have worked with.

MAMMALIAN SPERM

Recently methods have been devised for freezing and storing bull sperm for long periods of time. Currently, artificial insemination, using sperm from a few superior bulls, is common practice in the dairy industry. A suspension of bull sperm will be available for examination. Put a drop or two on a microscope slide, cover with a cover slip, and observe immediately under the compound microscope. Do not allow the preparation to dry out.

GENETICS EXPERIMENT

(continued: second week)

During a lull, make the new matings in your *Drosophila* experiment. The flies now in the vials are the F_1 generation. Etherize them carefully and examine them under the dissecting microscope. Record the phenotype of every fly. If they aren't as expected, consult an instructor; a mistake may have been made in the original mating.

Prepare two new sets of matings, placing 3 males and 3 females in each of two vials. Label the vials, and store them in the boxes until next week, when we will remove the parents. Two weeks from today, the F_2 generation will have emerged, and the results of the experiment will be analyzed.

Why have no precautions been taken to obtain virgin females for today's matings?

EQUIPMENT

Per student

small petri dish
2 droppers
microscope slides and cover slips
depression slide
dissecting microscope
compound microscope

sea water
prepared slides of sea urchin development
diluted suspensions of sea urchin eggs and sperm*
frozen bull sperm†
equipment for handling *Drosophila* is the same as for Exercise XXII
2 vials for *Drosophila*

*Detailed instructions for setting up the sea urchin experiment will be found in E. B. Harvey's excellent book, *The American Arbacia and Other Sea Urchins* (Princeton University Press, 1956). *Arbacia punctulata* is common on the East Coast, and *Strongylocentrotus purpuratus* or *S. franciscanus* on the West Coast. The latter forms, containing mature eggs and sperm, can be obtained during the fall and winter from the Pacific Bio-Marine Supply Co., P. O. Box 285, Venice, Cal.

†This must be kept at dry-ice or liquid-nitrogen temperature. Deep freezers are not cold enough to maintain such preparations. Sources of bull sperm will be found in the yellow pages of telephone directories, listed under "Livestock Breeders," or by contacting the county agent in agricultural communities, or any agricultural college.

XXIV DEVELOPMENT OF THE CHICK; CONTINUATION OF THE GENETICS EXPERIMENT

(Readings: J. D. Ebert, "The First Heartbeats," *Sci. Am.* 200, No. 3, 87–96, March 1959, Reprint No. 56. C. H. Waddington, "How Do Cells Differentiate?" *Sci. Am.* **189**, No. 3, 108–116, Sept. 1953, Reprint No. 45. See also the handsome photographs showing the progressive stages of chick development in the little book by E. Bosiger and J. M. Guilcher, *A Bird Is Born*, Sterling Pub. Co., 1959.)

The chick egg has been a classic object for the study of embryonic development for the last three-hundred years. It achieved this position in the great work of William Harvey (whom you already know as the discoverer of the circulation of the blood) on *The Generation of Animals*. This work contains on the title page the aphorism, *Ex ovo omnia*, "all life from the egg." The chick egg provides fine material for the analysis of development beyond the earliest stages, which have already passed before the egg is laid.

The egg is fertilized immediately after ovulation, as soon as it enters the oviduct. Usually five or six sperm enter, a common condition in the large eggs of certain amphibia, reptiles, and birds, though abnormal in most other animals. One sperm head eventually fuses with the egg nucleus; the others disintegrate.

At the time of sperm entry the egg nucleus is just entering its first maturation division, and must go on to complete its meiosis before the egg and sperm pronuclei fuse. Then cleavage begins, and goes through to early gastrulation within the hen, before the egg is laid. Also the walls of the oviduct secrete a layer of albumen around the egg, which serves later to float the embryo within the shell and provides it with an aqueous environment. The shell membranes and the porous limestone (calcium carbonate) shell are subsequently laid down by the shell gland. Only the yolk with its small disc of protoplasm represents the true ovarian egg. All the rest is accessory structure. All vertebrate embryos develop in an aqueous environment; and such eggs as this represent a device for bringing and maintaining an aqueous environment ashore—in a sense, an enclosed pond. How do you think the size of the yolk is correlated with the time it takes various types of egg to develop?

In today's experiment each student can examine an early stage in the development of the chick embryo. Record your observations

in a labeled sketch. Your partner will at the same time be examining an embryo at another stage, so that each pair of students will have a more-or-less complete picture of early development. Each student also will perform a test for cytochrome oxidase on his chick embryo.

Prepared slides of chick embryos will be available for examination under the microscope, representing stages of development both earlier and later than your live embryos. Examine them carefully, tracing the development of various parts of the embryo from stage to stage: the heart, brain, eye, limbs, musculature, and so on.

EARLY STAGES OF THE CHICK EMBRYO

Two students will work together on this experiment. Each pair will be given two eggs, one of which has been incubated at 38°C for 3 days and one for 5 days. Each egg is marked with the number of days of incubation.

In an egg left resting in one position for any length of time, the embryo has rotated so that the blastodisc is at the top, owing to the yolk being heavier. You cannot rely on this in the eggs given to you, and you should "candle" your egg to find where the embryo lies. This is conveniently done by holding the egg in front of a microscope lamp. Mark with a pencil the place on the egg where a shadow shows the embryo to lie, and keep this uppermost. Lay the egg in a petri dish, partly filled with warm Ringer solution, and carefully cut around the middle of the shell with scissors. Pick the shell off carefully. The unbroken yolk and embryo then will lie free in the Ringer solution. If a living embryo is not present, get another egg from the instructor. Use a dissecting microscope for observing the embryo.

Up to gastrulation, the chick follows much the same pattern of development as does the echinoderm egg. The differences are due mainly to the large amount of yolk in the chick egg, which crowds the protoplasm of the egg into a flat disc. Development proceeds primarily in this disc, rather than in the whole sphere of the egg as in a sea urchin. The embryonic heart begins to beat after 2 days, and some circulation of blood may be detected then. Anterior to the heart is the head, with its bulging, partly formed eyes. After 2 days the optic vesicles reach the optic cup stage and lenses form. Also the somites, precursors of the muscles, appear at about the same time, as blocks of tissue lined up in two rows along the trunk of the embryo. The vitelline blood vessels which carry food to the embryo emerge from the middle of the trunk and branch out over the yolk.

Three-day embryo. The embryo is bent back on itself, and lying on its side. Note the size of the head and the development of the heart. Two limb buds should be visible on each side of the embryo, as projecting lumps of tissue. The anterior limb buds will give rise to the wings, the posterior limb buds to the legs. Count the number of somites and note the development of the blood vessels surrounding the embryo. Record your observations in a labeled sketch.

Five-day embryo. The increased size and vascularity at this stage are obvious. It may help make fine details visible to rinse the embryo with several changes of warm Ringer solution. Blood vessels can be seen pressed close against the shell. What is their function? Toward the tail end of the embryo you should be able to see a fluid-filled sac, the allantois, which functions as a urinary bladder, and is one of the extraembryonic membranes. As the embryo metabolizes the food material of the yolk, waste products accumulate in this sac. The embryo is surrounded by the amnion, another extraembryonic membrane, but this is difficult to see after the egg has been opened.

CYTOCHROME OXIDASE IN THE CHICK EMBRYO

One can detect the presence of cytochrome oxidase in tissues with the so-called NADI

reagent. This is a mixture of alpha-*na*phthol and *di*methyl-para-phenylenediamine (hence "NADI"), which is oxidized to the blue pigment, indophenol blue, by cytochrome oxidase in the presence of oxygen.

Remove an embryo from the yolk and rinse it in warm saline solution. Draw off the saline, and replace it immediately with warm NADI reagent. Record the time. Now record the time for the first trace of blue color to appear, and continue to record its location and extent at three-minute intervals. Compare the results of your experiment with those of your partner on an older or younger embryo.

GENETICS EXPERIMENT

(continued: third week)

At some time during this period, go the next step in your *Drosophila* experiment. Last week, flies of the F_1 generation were mated. Today, these parent flies should be removed from the vials and disposed of in the morgues. Then replace the vials in the boxes for further incubation. Next week this experiment will be completed. The F_2 generation of adults will have emerged, and the results of the experiment can be analyzed.

EQUIPMENT

equipment for handling *Drosophila* as in Exercise XXII

fertile eggs incubated for 3 and 5 days (1 per student)

prepared slides of chick development

dissecting scope

petri dish

ringer solution, kept at 37°C (0.9% NaCl may be substituted for ringer solution if there is no desire to keep the embryo alive for an extensive period)

NADI reagent

Preparation of NADI reagent. This should be prepared just prior to use. Combine equal parts of 0.01-*M* alpha-naphthol, 0.01-*M* dimethyl-*p*-phenylenediamine (PPD), and phosphate buffer, pH 5.8. The alpha-naphthol is made by dissolving 1.44 gm in 1 liter of physiological saline (0.9% NaCl solution). Heat to dissolve. The PPD solution contains 1.36 gm in 1 liter of physiological saline. The phosphate buffer is a mixture of Na_2HPO_4 (9.5 gm/l distilled water), and KH_2PO_4 (9.07 gm/l distilled water), mixed in the proportions 7.8 mm Na_2HPO_4 solution:92.2 mm KH_2PO_4 solution.

XXV COMPLETION OF THE GENETICS EXPERIMENT

The adult F_2 flies in the dumpy-ebony experiment have now emerged, and this laboratory period will be devoted to examining them and evaluating the results. Since the flies will not be needed again, they may be overetherized before counting. The more flies counted, the more reliable the results will be.

Eight possible classes of flies can be distinguished from the crosses you have made: wild-type males, ebony males, dumpy males, ebony-dumpy males, and the same four classes of females. Determine the number of flies in each of the eight categories. From this you can tell whether the mutant genes are dominant or recessive, linked or not, and sex-linked or not.

After all the students have finished their counts, all the results will be summed up to give class totals, which can be treated as one large experiment.

Consider the dumpy : wild-type and ebony: wild-type ratios separately. What are they?

Make a diagram showing the genotypes of members of the parental generation, the F_1 generation, and the F_2 generation.

CHROMOSOME MAPPING: A THREE-GENE EXPERIMENT

The dumpy-ebony cross has illustrated simple segregation and independent assortment. To demonstrate a more complex situation in *Drosophila* genetics, you will work with a hatching generation in which three genes are segregated: apricot, cut, and bar. *Apricot* refers to the eye color which is much lighter than the wild-type red; *cut* to a marginal cleft in the wing; and *bar* to the eye shape: in the male the eye is restricted to a narrow vertical bar and in the female to a kidney-shaped slit. These characteristics are easily spotted. The symbol for apricot is w^a (since apricot is an allele of white); for cut it is *ct*, and *B* stands for bar.

Examine your flies (you may overetherize them) and record the various combinations of these genes and wild-type genes separately for the sexes.

There are eight possible phenotypes:

The parental types:

+	+	*B*	red eye, normal wing, bar eye
w^a	*ct*	+	apricot, cut wing, normal eye

Single crossovers in region I:

w^a	*ct*	*B*	apricot, cut wing, bar eye
+	+	+	completely wild-type

Single crossovers in region II:

w^a	+	*B*	apricot, normal wing, bar eye
+	*ct*	+	red eye, cut wing, normal eye

Possible double crossovers:

w^a	+	+	apricot, normal wing, normal eye
+	*ct*	*B*	red eye, cut wing, bar eye

Are all genes on the same chromosome? How do you know? If they do appear linked, calculate the percentage of crossing over between them. Prepare a map indicating relative positions of these genes on the chromosome(s). Diagram two generations of crosses giving rise to these offspring.

Interference of crossing over in one region with crossing over in another region can be tested in the following way:

$$\text{Coincidence} = \frac{\%\text{ double crossovers}}{\left\{\begin{array}{l}(\%\text{ crossovers in region I}) \\ \times (\%\text{ crossovers in region II})\end{array}\right\}}$$

(The denominator is the percentage of double crossovers that is expected.)

If the coincidence is less than 1.0, crossing over in one region interferes with that in another. One sometimes expresses what is called the "interference" as (1 — coincidence). Is there interference in this test cross?

PROBABILITY IN GENETICS

The interpretation of breeding experiments in genetics often requires statistical analysis; without the use of statistics it is sometimes impossible to decide whether the results of an experiment agree with those predicted by theory. A thorough treatment of the mathematics of genetics is beyond the scope of this course, but it will be helpful to consider a few elementary principles of probability in interpreting the *Drosophila* experiment and in understanding many aspects of segregation of genes.

The probability (P) that some event (x) will occur can be represented by a fraction between 0 and 1. This fraction is the proportion of times the event occurs (m) in a very large number of trials (n), *or*

$$P_x = \frac{m_x}{n_x}.$$

When in a very large number of trials, every trial yields the event, then $m = n$ and $P = 1$; the event is inevitable. When the event does not occur at all in a very large number of trials, $P = 0$; the event is impossible. Everything that happens has a probability that lies between these limits. The nearer P_x is to 1, the more probable the event.

Probability values are theoretical; they are merely mathematical expressions of expectations. It is necessary to perform a very large number of trials, and for an event to occur many times, for the observed frequency of successes to equal the probability. That is, the more trials and more often an event takes place, the more closely the proportion of successes will approach P_x.

To illustrate this, perform the following tests:

(a) Flip a coin four times and record the number of heads and of tails; repeat this four times. Note the variation in results.

(b) Flip the coin 10 times and again record the number of heads and of tails.

(c) Flip the coin 50 times and record the results.

(d) If you have time, extend this to 100 or more flips.

(e) Sum up the totals for heads and tails from (a), (b), (c), and (d) above.

(f) Calculate the ratio of heads (or tails) to the total number of tosses in each of (a), (b), (c), (d), and (e) above.

Just from the shape of a coin we expect the probability of a head (or tail) coming up on any flip to be about 0.5. Which coin-flipping test above provides the most reliable agreement with the theoretical value of P?

Simultaneous occurrence of independent events

The probability that several independent events will occur together is equal to the product of their separate probabilities, or

$$P_{x,y,z\ldots} = P_x \times P_y \times P_z\ldots$$

For instance, when two dice are tossed the

probability of "box cars" is the product of the probabilities of turning up a six on each die separately (1/6), *or* $P = 1/6 \times 1/6 = 1/36$.

Genetics makes frequent use of this principle. At fertilization an egg and a sperm combine randomly. For any genetic trait, the egg may contain either a dominant or a recessive gene, and likewise the sperm may contain either a dominant or a recessive gene. If one considers that the probability of an egg containing a dominant (or recessive) gene is 0.5, the same is true for the sperm. The probability, then, that the fertilized egg (zygote) will contain two dominant genes is the product 0.5×0.5, *or* 0.25. That is, in a suitably large population of offspring, 25% will carry two dominant genes (homozygous dominant). Likewise, 25% can be expected to carry two recessive genes (homozygous recessive). Another 25% of the offspring will receive a dominant gene from the father and a recessive from the mother; in the final 25% this is reversed, and a recessive gene will come from the father and a dominant from the mother. Thus 50% of the offspring should possess one dominant and one recessive gene (heterozygous).

Further coin-tossing tests help to illustrate the probability of occurrence of joint independent events such as these.

(a) Toss two coins at a time 12 times, and record the results: (h, h); (h, t); (t, t). Now using the principle that $P_{x,y} = P_x \times P_y$, calculate the probability of each paired outcome (2 heads, 2 tails, or a head and a tail). How closely do the results agree with the theoretical prediction? You might try tossing the two coins 100 times to see if the agreement is better.

(b) Repeat the above test tossing three coins 16 times. Calculate the probability of each combination: (h, h, h); (h, h, t); (h, t, t); and (t, t, t).

(c) Can you derive a general relationship that could be used to predict the results when n coins are tossed together a large number of times?

In a family with five children what is the probability that all will be daughters? that all will be of the same sex? (This is a problem in either-or probability. Whereas the probability that several events will all happen together is the *product* of their several probabilities, the probability that any one of several possible events will occur is the *sum* of their separate probabilities.)

For a more complete treatment of the use of statistical methods in heredity, consult any modern textbook of genetics. The chapter on "Statistical Inference in Genetics" in *Principles of Genetics*, by Sinnott, Dunn, and Dobzhansky, 5th ed., McGraw-Hill, 1958, is particularly recommended.

EQUIPMENT

equipment for handling *Drosophila* as in Exercise XXII

a hatching generation of *Drosophila* to illustrate sex-linkage. Although apricot, cut, and bar have been used as markers in the exercise here, many others will serve. Details can be found in any genetics text or in the *Drosophila Guide* mentioned in Exercise XXII.

XXVI SENSORY RECEPTORS

(Readings: G. von Bekesy, "The Ear," *Sci. Am.* **197**, No. 2, 66–78, Aug. 1957, Reprint No. 44. G. Wald, "Eye and Camera," *Sci. Am.* **183**, No. 2, 32–40, Aug. 1950, Reprint No. 46. W. Loewenstein, "Biological Transducers," *Sci. Am.* **203**, No. 2, 98–108, Aug. 1960, Reprint No. 70. S. P. T., pp. 195–208. Weisz, pp. 480–495. Villee, pp. 373–386.)

All that we know we learn through our sense organs. They are our ultimate instruments for exploring the environment. It is of the highest importance that we understand what kind of instruments they are, what they can do, and where they fail.

The anatomical unit of every receptor system is the single receptor cell or end-organ, particularly sensitive to one kind of stimulus, and giving rise to one quality of sensation in the brain. The effect of the stimulus upon such an end-organ is a depolarization ("generator potential"), in most cases long-lasting compared with the depolarizations that stimuli excite in nerve or muscle fibers—which in turn causes the firing of the attached nerve fiber. In some instances, as for example in touch spots, there may be only one or two all-or-nothing discharges in the nerve fiber in response to each stimulus. In most receptor systems, however, the depolarization of the end-organ lasts relatively long, and results in a long burst of all-or-nothing responses in the attached nerve fiber, which may cease after a time though the stimulus continues (e.g., smell), or may go on as long as the stimulus lasts, as in vision.

TOUCH

The skin contains a wide variety of end-organs, specific for pain, heat, cold, pressure, and touch. Touch receptors are of two kinds: bulbous arrangements of cells enclosing the naked terminal twigs of a sensory nerve fiber (Pacinian and Meissner corpuscles), or the widely branching terminal arborization of such a nerve fiber around the basal bulb or "root" of a hair ("hair crown"). With very small stimuli one can map out the locations of the sharply localized points at which stimulation conveys any one of the skin sensations.

We shall map out the touch spots in various areas of skin in this way, using a bristle as stimulus. This will demonstrate a general condition of all receptor systems—that the receptors form a discontinuous mosaic of isolated sensitive points, relatively coarse in the case of touch, and

varying greatly from one area of skin to another. Our sense of spatial continuity—of the smoothness of a surface—as well as of pattern is conveyed by such discontinuous mosaics of receptors.

The capacity of a sensory surface for evaluating pattern is measured by determining the "two-point threshold," which is the smallest separation at which two point stimuli are perceived as two. This measures the density of receptors, since for two stimuli to be appreciated as two, they must excite two touch spots having at least one unexcited touch spot between them.

Perform the following experiments in pairs, one student, with eyes closed during each test, serving as subject, the other as experimenter and recorder.

Distribution of touch spots

With a pen outline a square hairless or shaved area about 3 cm on a side on the inner forearm. Explore this area by touching it lightly with the tip of a bristle, noting the points from which a distinct sensation of touch is felt. Mark each of them with a spot of ink. Draw a diagram of the area, showing the locations of the touch spots. Estimate the number per square centimeter.

Two-point thresholds

To test for these, the experimenter touches various points in a region of skin very lightly with one or both of the blunted points of a pair of dividers, in haphazard order. At each touch the subject reports the sensation as either "one" or "two."

At the start of each test adjust the separation of the dividers so that all double stimuli are reported as "two" and all single stimuli as "one." Then gradually lessen the separation until only about 8 in 10 reports are correct. The separation of the points in centimeters is then the approximate *minimum perceptible* separation, or two-point threshold. In some areas of skin this is much the same in all orientations of the dividers; in others it differs greatly.

Determine and record in a table the two-point thresholds for the upper arm (longitudinal), upper arm (transverse), forearm (longitudinal), forearm (transverse), back of hand, palm of hand, fingertip, and lips.

Calculate the number of receptors per square centimeter in each area tested, and enter this in the table. In those areas in which the two-point threshold is about the same in all orientations of the dividers, use the formula: $N/\text{cm}^2 = 4/L^2$, in which N is the number of touch spots and L is the two-point threshold in centimeters. This formula is based on the assumption that the two-point threshold represents twice the distance between neighboring touch spots. Why twice the distance?

For areas of skin in which the longitudinal two-point threshold (L_1) differs from the transverse (L_2), the density is $N/\text{cm}^2 = 4/(L_1 \times L_2)$.

How does the density of touch spots on the forearm, calculated from the two-point threshold, compare with the density you found by direct mapping?

TASTE

The special senses (sight, hearing, smell, taste) are associated with dense aggregates of receptors, concentrated in limited areas, some of them supplied with highly adapted accessory structures, such as in the eye and ear. The sense of taste is limited to the mucosa of the tongue and mouth. The receptors are clustered in "taste buds" and are of several types, each type mediating a primary taste. Since the receptors for the several primary tastes are not uniformly distributed over the sensory surface, their nature and distribution can be determined by applying various solutions to different regions of the tongue.

A solution may stimulate more than one kind of taste cell, resulting in a wide variety of taste sensations. Other senses frequently enter: a solution that is both bitter and hot may give rise simultaneously to sensations of bitterness, warmth, and perhaps pain. Very often also, the

same solutions stimulate the sense of smell, and taste and smell together give us composite sensations of flavor.

Experiment

Again, work in pairs, one student serving as subject, the other as experimenter. The experimenter should moisten small rolls of filter paper in each of the following solutions:

quinine sulphate,

5% sugar (sucrose),

10% sodium chloride,

1% acetic acid,

and, after shaking off excess liquid, apply each in turn with forceps to different regions of the tongue of the subject, for about 10 seconds. At each application the subject should report the sensation as "bitter," "sweet," "salt," "sour," or "none," and should rinse the mouth with water after each test.

On a diagram of the tongue mark each region from which sensation is reported, using a different symbol (circle, square, triangle, cross) for each primary taste.

To demonstrate the role of smell in deciding flavor, alternately place a bit of apple and a bit of onion on the subject's tongue, while he keeps his eyes closed and *holds his nose shut*. Can the subject distinguish them by taste alone?

Demonstration

Taste and smell are confined to mucous surfaces. The stimuli for these chemo-receptors are always substances in aqueous solution. For substances to reach our olfactory areas and stimulate smell sensations they must obviously be in the gaseous state, so that they can be inhaled; but in this case also these substances must dissolve in the layer of mucous that covers the olfactory patch before they can stimulate the smell receptors.

Animals which have wet skins apparently have such chemo-receptors distributed over large areas or the entire surface. This is true in general of fishes and amphibia.

Destroy the brain of a frog by pithing or by cutting off the head just at the angle of the jaws. Allow the frog to lie undisturbed for a time to recover from the shock. Now lay a bit of filter paper soaked in dilute acid on its flank, and observe what happens. Touch a similar piece of filter paper soaked in the same acid to your tongue. Note that to you this is a stimulus for taste, not for pain. Presumably it is the same kind of thing for the frog, and unlike us, the frog apparently can taste all over.

This response in the absence of the brain is an extraordinary demonstration of a complex spinal reflex. Here a mild stimulus, no more than "distasteful," evokes a reasonable and accurate response, all handled at the level of the spinal cord.

SENSORY JUDGMENTS OF INTENSITY: THE ESTIMATION OF WEIGHT

All measurements are ultimately sensory judgments of quantity. Yet the response of a receptor to a stimulus varies with duration and state of adaptation, so that sensory reports of intensity of stimulation are at best relative. In general, as regards intensity, our receptors permit only three kinds of measurement: (a) the absolute threshold: the strength of stimulus that just excites the sensation; (b) the intensities at which two stimuli seem just equal; (c) the intensities at which two stimuli are just perceptibly unequal. This last is the "difference threshold." It plays the same role in our estimation of intensity as the two-point threshold does in our estimation of space.

Beyond these judgments, all measurement ceases. So, for example, I can say accurately the intensity of light that is just visible, its absolute threshold; or how much I need to increase the intensity of a light to make it just perceptibly brighter, the difference threshold;

or that two lights are equal in brightness. What would it mean, however, for me to say that one light is 2.3 times as bright as another?

Weber (1834), experimenting initially with weights, discovered that the difference threshold varies with the intensity of the stimulus in a peculiar way that became known as Weber's law: the difference threshold (ΔI) is a constant fraction of the intensity of stimulus (I), that is, $\Delta I/I$ = constant. The ratio $\Delta I/I$ is called the Weber fraction. It is an inverse measure of the capacity to discriminate intensities; the larger this ratio, the poorer is the capacity for making such discriminations. Weber's law holds only approximately and over a limited range. The ratio $\Delta I/I$ remains approximately constant for many senses over the middle ranges of intensity, but rises at both the low and high extremes of intensity.

Estimation of weight

A weight held in the hand is supported by muscle tensions in the hand and arm. These stimulate tension receptors in the muscles and tendons, the reports of which help to guide the limb and also excite sensations. If two weights are successively lifted, there exists a minimal difference in weight such that one is judged just heavier than the other, the difference threshold. This may be determined for various weights, and the constancy of the Weber ratio tested.

Working in pairs, perform the following experiment. You have two 125-ml Erlenmeyer flasks. Mark one of them with a crayon, to be the test flask. Add water to both so as to bring them to equal weight at about 50 grams. The experimenter now hands the flasks to the subject, whose eyes are closed. The subject holds the flasks either cupped in his palms or with his fingers by the necks, but whichever way he chooses should be maintained throughout the experiment. For this first experiment the flasks should be held steady, and the subject says whether they feel equal or unequal in weight. Presumably they feel equal. Now the experimenter takes the flasks again, and adds water to the test flask in 2-ml portions, each time handing the flasks back to the subject, randomly mixing right and left, each time giving the subject all the time he needs to decide the relative weights. When the test flask feels just perceptibly heavier than the other, record the volume of water that was added to it. This is also the added weight, since 1 ml of water weighs 1 gram. The difference in weight is then the difference threshold for a weight of 50 grams.

Now repeat this procedure with the flasks initially made equal in weight at about 100, 200, and 500 grams (the latter two in 500-ml flasks) and tabulate the results.

Prepare a graph plotting the Weber ratio (Δ weight/lower weight) on the vertical axis against the lower weight on the horizontal axis.

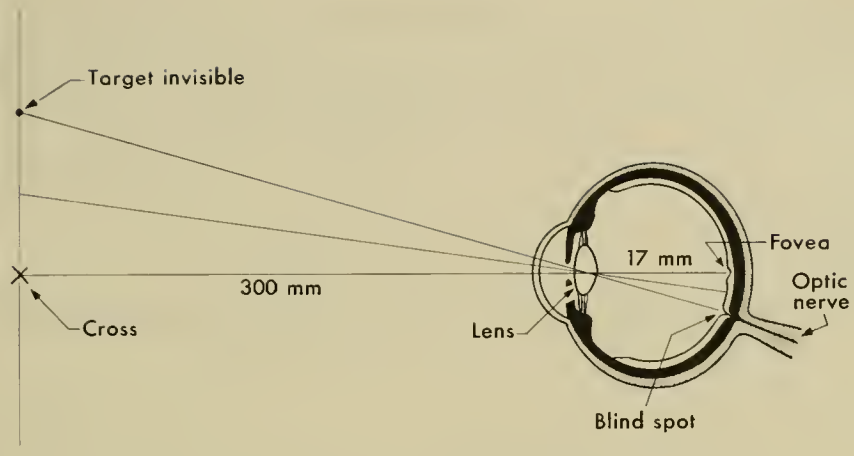
Repeat this experiment for at least one weight, or all the way through if you have time, with the subject wagging the flasks up and down as he estimates their weights. Do you find a difference in the Weber ratio? Motion in general produces a much stronger and more persistent excitation than a stationary stimulus. Why? (Recall the effectiveness of flickered as compared with steady light, in Exercise XX, p. 108.)

Has the Weber ratio remained approximately constant in your experiments? What do you conclude of the accuracy with which weights can be estimated? How do the Weber ratios of other subjects compare with yours? This last question illustrates one example of the "personal equation" that is involved in every type of sensory judgment.

VISION

The blind spot

The point at which the optic nerve leaves the retina is blind, since this area contains no visual receptors. Lay a sheet of blank white paper on the desk, and draw a small cross to the left of center. The subject, holding his left eye closed, should stare fixedly at the cross with his right eye 30 cm from it. (Staring fixedly at anything means holding its image within the central fovea



of the retina, so fixing its position on the retinal surface.) The experimenter, without jogging the paper, slowly advances a small target (a pencil point will do) into the subject's field of vision, starting about 2 to 4 inches to the right of the cross. There is a point at which the target disappears. The experimenter marks this point on the paper, and starts again from another angle. By repeating this performance, advancing the target from various angles around the cross, one can plot the entire boundary of the blind spot.

If there is time, repeat this for the left eye; this time, however, the target should be introduced at the left of the cross.

The accompanying figure shows diagrammatically the optics of this experiment. Study it carefully, and see that you understand everything in it, for it contains the essential elements of image formation in the eye. From the results of your experiment calculate the diameter of the blind spot in the eye, and also the distance of its center from the fixation point within the central fovea. This is done very easily, since the projected dimensions on the paper are to the dimensions on the retinal surface as the distance from the paper to the eye (300 mm) is to 17 mm, the focal length of the human eye.

Note that, like any other simple lens system, the eye inverts the images of all objects at which one looks, and equally inverts all spatial relations. Why then do we not see upside down, and wrong end to?

Retinal blood vessels

As a consequence of the way it develops embryologically, the vertebrate retina points *away from* the light. Light must pass through the entire thickness of the retina, including the retinal blood vessels, before reaching the visual receptors. The blood vessels therefore cast a continuous shadow upon the visual field; and the only reason we are not aware of this at all times is that one cannot continue to see any image that is fixed in position on the retinal surface. To make the blood vessels visible, all that is needed is to make their shadows *move*.

Make a small hole, about 1 mm across, in a card, and look through it at a brightly illuminated white surface, meanwhile giving the hole a rapid side-to-side or rotary motion. Shortly you should become aware of a delicate, lacy network, with a central open space, as though a hole were torn in it. The hole will move wherever the eye is fixated. The network represents the shadows of the retinal capillaries, the hole the central fovea, from which blood vessels are lacking.

Those of you who see this plainly might like to estimate the diameter of the fovea. This can be done by estimating the width of the image of the capillary-free area as projected on the white surface; from this and the distance of the surface from the eye, you can complete the calculation as you did above for the blind spot.

EQUIPMENT

Per laboratory

safety razor
filter paper
1% quinine sulfate
5% sucrose
10% sodium chloride
1% acetic acid
onions and apples
frog
pan balance

Per pair of students

bristle
dividers
2 125-ml Erlenmeyer flasks
marking pencil
pipet
2 500-ml flasks
sheet of white paper
3″ × 5″ white card

OUTLINE FOR THE INSTRUCTOR ON THE PREPARATION FOR MICROBIOLOGICAL EXPERIMENTS (EXERCISES VI THROUGH IX)

APPENDIX A

A. MATERIALS

1. Glassware

Pyrex *culture tubes* without lips are preferable. They will be needed in three sizes: 13 × 100 mm, 16 × 150 mm, and 20 × 150 mm. Along with the 5-ml serological pipets and Erlenmeyer flasks, they may be obtained from any scientific supply house.

Dropper pipets of sufficient length for transfers from tubes (about six inches) are difficult to obtain commercially and probably will have to be made up. Alternatively, 1-ml serological pipets or Pasteur pipets may be used.

Sterile disposable *petri dishes*, 15 × 100 mm, available from Falcon Plastics, 5500 West 83rd St., Los Angeles 45, or from Scientific Products, 1210 Leon Place, Evanston, Illinois, at a cost of about five cents apiece, are recommended since their use obviates the need for much tedious cleaning, washing, and sterilizing, and also allows the students to take plates home with them to observe growth.

2. Media and Chemicals

Ready-mixed media such as nutrient broth, nutrient agar, and tryptose blood agar base can be obtained from Difco Laboratories, Detroit 1, Michigan. They should be made up according to the directions on the bottles.

Of the constituents for the Pneumococcal media, Casamino acids, tryptone, yeast extract, and brain-heart infusion are obtained from Difco. All other organic materials including vitamins, amino acids, sugars, streptomycin, deoxycholic acid, methylene blue, serum albumin, and sterile horse blood may be obtained from Nutritional Biochemicals, Cleveland, Ohio. Fresh yeast can be procured from Standard Brands, Inc.

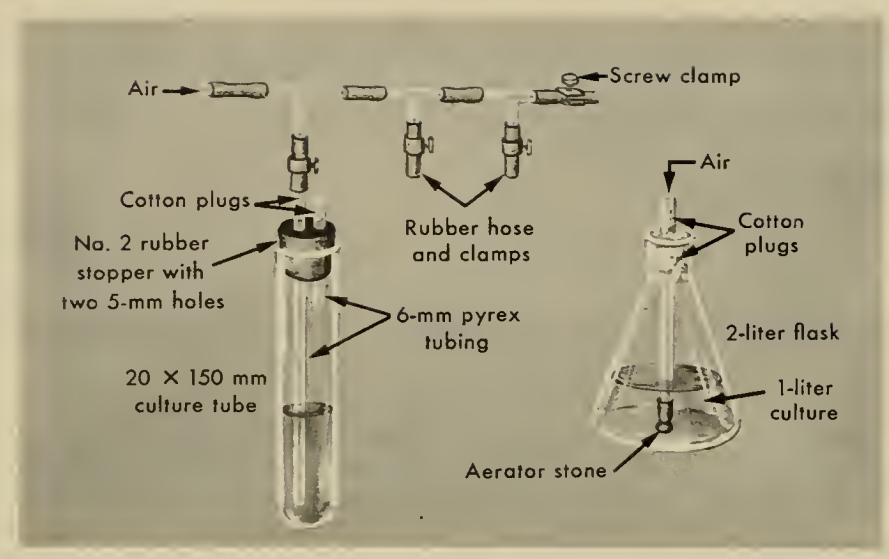
Inorganic chemicals, reagent grade, are available from any chemical supply house. Antifoam may be obtained from the Dow Chemical Company.

3. Miscellaneous

a. Constant-temperature equipment

Water baths may be rigged up from parts which can be obtained at relatively low cost from an aquarium supply house. A tank 16″ × 10″ × 12″ deep, fitted with a 100-watt thermostat aquarium heater and a 100-watt constant heating element and either an air line or aquarium bubbler for stirring, will provide room for eight students. However, if at all possible, it is recommended that water baths or their components be obtained from scientific research supply houses in order to achieve more reliable temperature regulation.

Two neoprene-coated test-tube racks with holes large enough to accommodate tubes 20 mm in diameter (obtainable from Emil Greiner Co., New York City) may be supported in the baths by means of platforms made of $\frac{1}{4}$″ mesh-wire screening.



An incubator cabinet of moderate size, available from any scientific supply house, is useful for the preparation of large quantities of culture and is essential for the incubation of plates containing *Pneumococcus*.

b. Aeration

An aerator assembly, leading from either a compressed air line or an aquarium bubbler (alternatively, suction may be used to drive the aerator), composed as shown in the diagram, provides for aeration of four student cultures.

For aeration of large volumes of culture (100 ml to 1000 ml), an aerator stone (available from Fisher Scientific Co.) attached by gum rubber tubing to a pyrex tube plugged at the opposite end with cotton should be used.

c. Ultraviolet irradiation

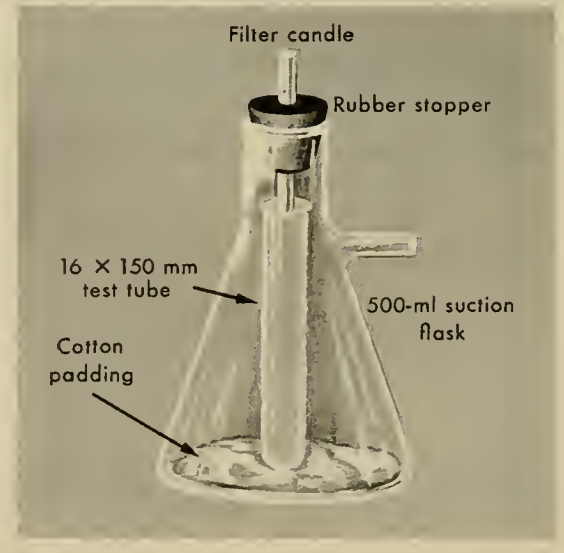
A satisfactory source of ultraviolet light is a General Electric 15-watt germicidal lamp installed in an ordinary fluorescent desk fixture.

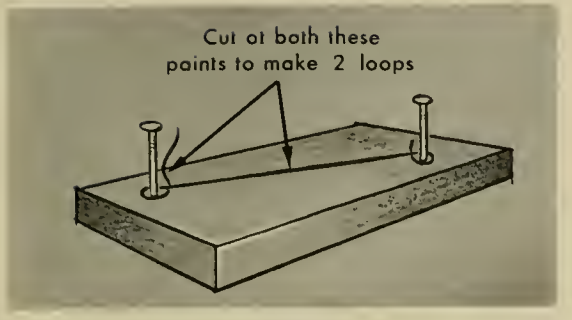
To avoid injury to the eyes, students should wear safety glasses of either glass or plastic.

d. Filtration

Seitz filters and filter assembles for preparative sterilization of solutions containing heat-labile material may be purchased from any scientific supply house.

Porcelain candle filters, made by Coors, size 10 mm × 55 mm, porosity No. 5, for use in the virus filterability experiment may be obtained from Arthur H. Thomas Co., Philadelphia, Pa. The filters should be inserted into rubber stoppers to fit into 500-ml suction flasks and permanently marked for use in phage or bacterial filtration. The filtration assembly should look as shown in the diagram.





e. Bacteriological loops

These should be made from nichrome wire, No. 23 gauge. The loop should be about $\frac{1}{8}''$ in diameter; it is convenient to form such loops by bending the wire around two nails of appropriate size imbedded in a wood block, as shown in the diagram.

The wire is either inserted into a commercially available loop holder or fused into the end of a 4″ length of thick-walled capillary tubing to serve as a handle. Alternatively loops may be purchased already assembled.

B. WASHING OF GLASSWARE

All glassware should be thoroughly washed with detergent and rinsed at least five times in order to remove traces of detergent which might be toxic for the bacteria. It is not necessary to rinse this glassware with distilled water, though the latter should be used for making up all media and solutions. It is advisable that the students be taught to do as much of the washing and plugging of pipets and aeration tubes as possible.

C. STERILIZATION

In general, vessels containing liquids, or assemblies containing rubber parts, should be autoclaved at 120°C (15-lb pressure) for 15 min. Large volumes (greater than 200 ml), particularly of viscous liquids such as agar, should be autoclaved for longer periods (30 min to 1 hr).

Empty glassware should be dry-sterilized in an oven at 160°C for at least 90 min.

Serological pipets, culture tubes, flasks, and aerators must be plugged with nonabsorbent cotton before sterilization. If proper cans are not available, serological pipets, dropper pipets, and aerators may be wrapped in bunches with aluminum foil so that sterility is preserved on removal.

Sugar solutions are best sterilized by heating for 20 min in a boiling water bath or for one hour in an inspissator (steam box). However, concentrated glucose solutions (10%) may be wet-autoclaved the same as media and then measured out after cooling.

Solutions of labile materials must be sterilized by filtration.

D. SOURCES OF CULTURES

Cultures of *Bacillus megatherium*, *Serratia marcescens*, and *Escherichia coli B* may be obtained from the American Type Culture Collection, 212 M Street, N.W., Washington, D.C. They should be propagated every two months by streaking a sample of the old culture onto the surface of a fresh nutrient agar slant and incubating until growth is completed, after which the cultures should be conserved in the cold. Agar slants are prepared by adding about 5 ml of liquid nutrient agar to a sterile screw-cap vial, about 16 mm × 150 mm in dimensions. Tilting the tube before solidification results in a slant surface of considerable area, in a tube of small cross section, and hence relatively little risk of contamination.

Strains of *Pneumococcus* which are nonencapsulated or rough, and therefore nonpathogenic, must be used. A normal strain sensitive to streptomycin, as well as a streptomycin-resistant strain, can be obtained from universities or institutes carrying on research on the transformation of *Pneumococcus*. Among such institutions are The Rockefeller Institute, New York City; University of Colorado Medical Center, Denver, Colorado; Laboratoire de Génétique Physiologique, C.N.R.S., Gif-sur-Yvette (Seine-et-Oise), France; and Brookhaven National Laboratories, Upton, New York.

Pneumococcal strains are best propagated by growing an inoculum in the medium described in part E-3 of this outline, supplemented with $\frac{1}{20}$ volume of fresh yeast extract. (Fresh yeast extract is prepared by crumbling 1 pound of fresh yeast in 1 liter of water, bring to a boil, cooling, centrifuging, and sterile-filtering the supernatant.) The culture is grown at 37°C until visibly turbid; $\frac{1}{10}$ volume of sterile glycerol is added, and the culture is frozen at —20°C. Such frozen cultures retain their viability for three months to a year. Competent cultures of streptomycin-sensitive cells to be transformed are grown in the same fashion; they retain optimal transformability for a week or two.

Bacteriophage must be procured from laboratories doing research on bacteriophage. Most universities, medical schools, or research institutes could either supply the virus or else suggest where it could be obtained. The virus is propagated by addition of a sample to a culture of *E. coli B* in logarithmic growth at a density of about 10^8 cells/ml. Incubation is continued until the culture lyses. The resultant phage suspension may be kept sterile by addition of a drop of chloroform. (All chloroform must be removed by aeration before using the virus.) The concentration of particles may be determined by the method described in Exercise IX or, more accurately, by the agar layer technique. For details of this technique and other useful information on bacteriophage properties and handling, see M. H. Adams, *Bacteriophages*, New York, Interscience Publishers, Inc., 1959.

E. DETAILS OF PREPARATION*

1. Exercise VI

Agar plates. Disperse 350 gm of nutrient agar in 10.5 liters H_2O in six 2-liter Erlenmeyer flasks. Plug. Autoclave 40 min. Let flasks cool to about 60°C. Pour layer equivalent to 30 ml into each of 300 petri dishes. Store plates at room temperature.

Broth. Dissolve 40 gm of nutrient broth and 24 gm NaCl in 5 liters H_2O. Add a squirt of antifoam. Distribute: 200 ml in each of thirteen 500-ml flasks, 800 ml in a 2-liter flask containing a stone aerator, 300 ml in a 500-ml flask. Plug. Autoclave 20 min.

3% H_2O_2. Dilute 30 ml of 30% H_2O_2 (Superoxal) with 270 ml H_2O.

10% hydroxylamine. Dissolve 10 gm hydroxylamine hydrochloride in 50 ml H_2O. Add sufficient 10% NaOH to give pH 7. Add water to give a total volume of 100 ml.

Blood. Dilute 5 ml defibrinated horse blood with 95 ml NaCl, 0.85%. Refrigerate.

Cultures. Inoculate loopful of *Serratia marcescens* from agar slant into 800 ml of broth. Incubate at 37°C with gentle aeration for 10–20 hours. Culture should be heavy.

Transfer 3 ml with aerator to 300-ml broth 4 to 6 hours before class begins. Aerate at 37°C. Divide up the rest of the culture into flasks to be used as the "old culture." Refrigerate these until class begins. Just before class, distribute the "young culture" (transfer made 4–6 hours earlier) in sterile fashion.

2. Exercise VII

Agar plates. See E-1 above. Prepare 500 plates.

Broth. See E-1 above. Two 200-ml portions with aerators for growing cultures of *Serratia marcescens* and *Bacillus megatherium* are needed.

Cultures. Inoculate broth with a loopful from agar slants of *Serratia marcescens* and *Bacillus megatherium* and grow the two cultures overnight at 37°C with aeration. (*Note:* When grown at temperatures over 30°C, *Serratia marcescens* may not develop its characteristic red pigment.)

Alkaline methylene blue. Dissolve 1 gm methylene blue in 100 ml of 95% alcohol. Add 300 ml 0.01% KOH.

* For a class of 100 students.

3. Exercise VIII

Blood agar streptomycin plates. Prepare four 1-liter batches in 2-liter flasks. Disperse 35 gm tryptose blood agar in 1 liter H_2O. Autoclave 30 min at 120°C. Cool to 50°C. Add, per flask:

5 ml sucrose, 20%, sterilized by heating 15 min in boiling water,
5 ml streptomycin sulfate, 10 mg/ml, sterilized by filtration,
20 ml sterile horse blood.

Pour 120 plates, each containing about 30 ml.

Growth medium for streptomycin-resistant cells. Prepare three 1.5-liter batches in 2-liter flasks. To 1.5 liters H_2O add

10 gm brain-heart infusion,
10 gm Difco yeast extract,
10 gm Casamino acids,
10 gm tryptone,
3 gm glucose.

Adjust pH to about 7.5 with 10% NaOH. Autoclave.

Pneumococcal medium for competent streptomycin-sensitive cells—basal. Dissolve:

36 mg tryptophan,
200 mg cycteine-HCl,
12 gm sodium acetate,
30 gm Casamino acids,
51 gm K_2HPO_4

in 6 liters H_2O. Distribute: 200 ml in each of sixteen 500-ml flasks, and 900 ml in each of three 2-liter flasks. Plug. Autoclave 15 min. Store at room temperature.

Addition mix. Dissolve in 200 ml H_2O the following substances (this will make sufficient mix for 4 liters of basal medium):

2 gm $MgCl_2 \cdot 6H_2O$,
10 mg $CaCl_2$,
100 μg $MnSO_4 \cdot 4H_2O$,
0.8 μg biotin,
0.8 mg nicotinic acid,
0.8 mg pyridoxine·HCl
0.8 mg thiamine·HCl,
0.4 mg riboflavin,
2.4 mg calcium pantothenate,
2 μg $FeSO_4 \cdot 7H_2O$,
2 μg $CuSO_4 \cdot 5H_2O$,
2 μg $ZnSO_4 \cdot 7H_2O$,
20 mg choline,
40 mg glutamine,
200 mg asparagine,
20 mg adenine,
2 gm serum albumin (Armour Fraction V).

Adjust pH to 7. Sterilize by filtration.

Citrate-saline. Dissolve 6 gm NaCl and 20 gm sodium citrate in 700 ml H_2O.

Deoxycholate solution. Dissolve 5 gm deoxycholic acid in 80 ml H_2O by addition of 10% NaOH to bring pH to about 7.5. Add water to bring to 100 ml.

Growth of streptomycin-resistant cells. Inoculate 2 drops of thawed culture of SR into each of three flasks. Incubate without aeration for 15 hours at 37°C or until growth is maximal. Refrigerate until 1–2 hours before class. Centrifuge cells. Resuspend in 700 ml citrate-saline. (If growth has been poor, resuspend in less volume.)

(*Note:* All cultures should be examined microscopically in order to determine that they are not grossly contaminated. It may be advisable to grow cultures in duplicate to allow for those discarded because of contamination.)

Growth of competent streptomycin-resistant cells. Add 5 ml addition mix and 1 ml autoclaved 20% glucose to every 100 ml of basal medium to form the complete medium. Inoculate with a drop or two of the frozen culture of streptomycin-resistant cells and incubate as above. Dispense convenient volumes into sterile tubes for use in class. These must be kept in ice.

4. Exercise IX

***E. coli* culture.** Inoculate loopful of *E. coli B* from agar slant into 20 ml nutrient broth. Aerate at 37°C overnight. Inoculate 10 ml of

this culture into 1.3 liters of nutrient broth. Aerate at 37°C for 4 hours or until culture contains from 5×10^8 to 1×10^9 cells per ml. Use this young culture.

Phage suspension. Grow 20 ml of culture of *E. coli B* with aeration at 37°C to a density of about 2×10^8 cells/ml. Infect with a drop of phage suspension containing 10^6 to 10^8 infective units. Continue incubation until visible lysis occurs. Determine infective titer. Dilute as called for.

Soft agar.

bacto-tryptone, 10 gm,
Difco agar, 7 gm,
sodium chloride, 5 gm,
water to 1 liter.

Nutrient agar plates. 2.3% nutrient agar in distilled water.

NOTES TO THE INSTRUCTOR ON THE ELECTRONIC EQUIPMENT USED IN EXERCISE XVIII

APPENDIX B

The most satisfactory equipment now available for teaching electrophysiology to students at any level is the Tektronix "*160* series" of instruments, manufactured by Tektronix, Inc., Beaverton, Oregon. This equipment, designed originally for teaching medical school neurophysiology, has proved to be extremely dependable, easy to operate, and of high research quality. For the experiments chosen here, we purchased the following pieces of equipment (prices as of early 1961):

Type *360* Tektronix Indicator Unit	at $250.00
Type *162* Tektronix Waveform Generator	at $125.00
Type *161* Tektronix Pulse Generator	at $125.00
Type *160A* Tektronix Power Supply	at $175.00
Type *122* Tektronix Preamplifier	at $130.00
Type *125* Tektronix Amplifier Power Supply	at $250.00*

The waveform generator provides the proper voltage to drive the horizontal sweep (100 μsec to 10 sec) of the electron beam of the indicator, and also to trigger the pulse generator. Pulse stimuli of 0 to 50 volts amplitude and 10 μsec to 0.1 sec duration from the pulse generator are therefore always synchronized with the sweep of the horizontal beam of the indicator, thereby facilitating the observation of electrically evoked responses.

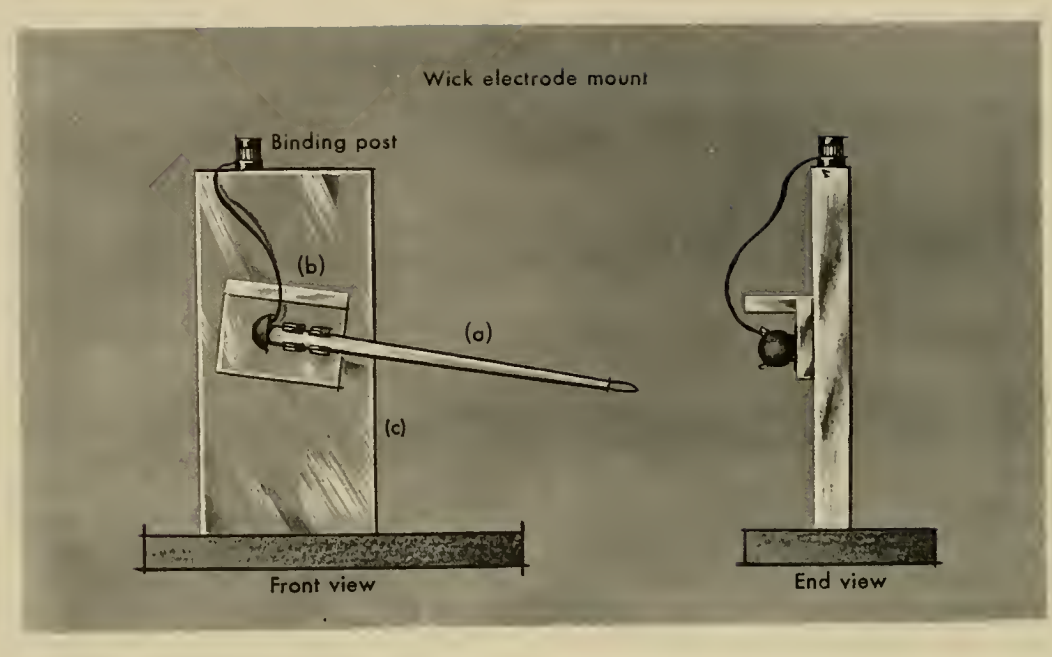
The *122* preamplifier (a-c, 0.16 cycles to 40 kilocycles) provides amplification of 100 or 1000, and together with the *360* indicator unit provides a sensitivity of 50 μv/cm.

The *160A* power supply provides the required voltages and currents for the *360* indicator unit, the *162* waveform generator, and the *161* pulse generator; while the *125* preamplifier power supply powers up to four *122* preamplifiers. The need for batteries with this equipment is therefore eliminated; all the current needed is provided by a 110- or 220-volt a-c wall outlet.

The instruments may be attractively and conveniently mounted in frames of standard rack-mount dimensions, and either placed in open racks or cabinets. We have frame-mounted the *360* indicator, *161* pulse generator, *162* waveform generator, and *160A* power supply together; and with a rack-mounting model *122* preamplifier housed our instruments in a Bud cabinet (model CR 1736, Bud Radio Co., Cleveland, Ohio). Each *125* amplifier power supply is placed conveniently to four such setups, and connected via cables with the amplifiers.

The nerve chambers which we have found satisfactory were purchased from the Harvard Apparatus Company, Dover, Mass. ($13.75 a-piece). The wick electrodes used for recording the electrical activity of the Limulus eye were designed for this experiment, but undoubtedly would be satisfactory for any experiment in which wick electrodes were required.

* One *125* preamplifier power supply serves for four setups.



As shown on the diagram, heavy cotton thread extending through a tapering glass pipet (a) filled with sea water provides a convenient wick. The pipet is fastened with small fuse clips to a small piece of lucite (b) ($2'' \times 1'' \times \frac{1}{8}''$), which is mounted on the large lucite base (c) ($3'' \times 6'' \times \frac{3}{8}''$) with heavy stopcock grease. The pipet is thereby readily movable with regard to the base and can be positioned in any desired direction. A silver-silver chloride wire runs through the pipet and attaches to a binding post screwed into the top of the base. The rear end of the pipet is sealed off with a rubber cap, preventing the pipet from drying out rapidly. When not in use, the wick end of the pipet should be immersed in sea water.

We have not found it necessary to shield the input cables for these experiments; but when using the exposed wick electrodes, a copper shielding cage is necessary to enclose the preparation. We have attached standard banana plugs on all our leads for convenience in connecting the instruments both to nerve chambers and electrodes.

SUPPLEMENTARY EXPERIMENTS ON THE CHEMICAL COMPONENTS OF CELLS: THE BIOCHEMISTRY OF MILK*

APPENDIX C

PART 1

(Reading: S. T. P., pp., 70–85; 121–123.)

Milk, the cellular product of a mother, contains everything needed for the cellular growth of a baby: proteins, fats, sugars, mineral salts, and vitamins.

In this exercise we will work with skimmed milk, since the isolation and properties of the fatty components are already familiar to you in the form of butter. Incidentally, one of the most general properties of a fat is that it makes a nonvolatile grease stain on paper; but you certainly don't need to do that in the laboratory.

Proteins are the large molecules of which the cell principally builds its structure and machinery. They are composed of chains of amino acids. We shall isolate a protein called casein by neutralizing the negative charges on the casein which cause the molecules to repel each other. This will be done by adding acid, that is, a source of positively charged hydrogen ions (protons). When the hydrogen ions bind to the negatively charged casein molecules, the latter no longer repel one another, and they begin to aggregate, causing precipitation of the protein, and permitting its filtration.

After the casein is removed, the other proteins will be coagulated by heating and evaporating the solution. Heat causes proteins to lose their delicate normal structure; the normally coiled chains of amino acids unwind. If there are enough molecules in close contact, chains of different molecules intertwine, giving rise to an insoluble coagulum. How does evaporation of the solution enhance this process? (When a protein is thrown out of solution relatively unchanged, so that it can be redissolved, we call that *precipitation*. In *coagulation* its structure has been unraveled irreversibly, and it cannot be redissolved.)

Two important mineral ions of milk are calcium (Ca^{++}) and phosphate ($PO_4^{\equiv}$). Both of them are essential in the formation of bone, and calcium ions are necessary also for such diverse biological processes as blood clotting and muscle action. On continued evaporation of the milk, calcium and phosphate ions come into closer and closer contact until they reach such a concentration that attractive forces between them cause them to precipitate as the salt, calcium phosphate.

Sugars serve living cells as sources of fuel and material, performing as middlemen which enable chemical reactions carried out in one cell or organism to support activity in another cell or organism. They are small molecules composed of carbon, hydrogen, and oxygen atoms, in the proportion $(CH_2O)_n$. The sugar present in milk is lactose. It will be isolated by adding the residue from the milk to acetone. Lactose is insoluble in acetone; that is, the lactose molecules tend to stick together in an orderly array rather than float about singly in the acetone. Keeping the solution cold hastens crystallization

* This material covers two laboratory sessions.

by reducing the tendency of the molecules to move around.

Specific tests enable us to characterize the isolated components. The reagent in the biuret test gives a color with substances composed of linked amino acids. Similarly, Benedict solution reacts with aldehyde groups (—HC=O) found in sugars.

Differentiation between large and small molecules can be accomplished by placing the milk in a sac which has pores so fine that only small molecules may pass through. The wall of such a sac is thus a semipermeable membrane, and the process of ultrafiltering mixtures of large and small molecules through such a semipermeable membrane is called dialysis.

The amino acid composition of casein will be examined by allowing a proteolytic enzyme to break it down. The proteolytic enzyme is a protein isolated from the pancreas gland, which forms important enzymes for the digestive system. It is capable of splitting the links between the amino acids in a protein. The breakdown products of casein will be analyzed by paper chromatography.

ON THE SEPARATION OF COMPOUNDS

Organic chemistry began as the chemistry of carbon compounds that occur in, or are produced by, living organisms. So it remained for a time until it was discovered that it was possible to synthesize innumerable unnatural compounds of carbon, and organic chemistry went its own way to leave the naturally occurring compounds in the realm of biological chemistry.

The major effort in biochemistry was devoted for many years to its medical aspects, and methods were developed for the qualitative and quantitative determination of constituents of milk, saliva, blood, urine, feces, and gastric juices. These are materials easily obtainable for analysis, and valuable for the clinical diagnosis of human diseases.

Many of the methods developed in this connection, however, are not exact, since chemical tests which may be satisfactory for the detection of a substance in one biological fluid or extract are unsatisfactory in other preparations. The difficulty is that such complicated mixtures vary greatly in chemical composition (even within the different tissues of the same organism), and interfering compounds occur in some preparations though not in others. For this reason it has been necessary to devise procedures to separate mixtures into simpler fractions, in the best case containing pure or nearly pure compounds.

One of the most rapid and convenient methods for doing this is paper chromatography. This method in combination with the use of relatively specific chemical tests provides a general scheme for evaluating the chemical composition of all kinds of complex mixtures of biological origin.

You will use this technique, not only to reveal the complexity of a large natural molecule, the protein casein, but also to identify a single substance among a variety of possibilities.

The movement of substances on filter paper depends on their solubility in the developing solvent, adsorption on the paper, and often on partition between two solvents. Some substances can be separated fairly well in distilled water, but mixtures of water with various organic solvents are usually more satisfactory. The aqueous portion usually contains acid or base to minimize the existence of more than one ionized form of any dissociable substance in the sample. This is not always necessary, and neutral or buffered solvents are frequently used. The volatile alcohols (methanol, propanol, butanol) and acids (formic, acetic, hydrochloric) are convenient, and ammonia is the most generally satisfactory base.

EXPERIMENTAL PROCEDURES

Measure 200 ml of skimmed milk into a 400-ml beaker. Add hydrochloric acid drop by drop until a precipitate of casein appears. About 50 drops will be needed. Stir with a glass rod while adding the acid. Add 40 drops,

then titrate drop by drop, touching the glass rod to the indicator paper. Let the precipitate settle for 5 minutes. Filter it through cloth as follows: place a piece of cloth over a beaker; depress the middle. Pour in the suspension, slowly. When the flow slows down, shape the cloth into a bag, and squeeze as much of the excess liquid into the beaker as possible.

Add one marble chip to the filtrate in the beaker, mark the level of the liquid with a wax pencil, and boil gently over the flame of a bunsen burner until the volume of liquid is reduced to a little less than half. (The marble chip will prevent bumping during the boiling.)

Meanwhile, continue working with the casein. With the precipitate still in the cloth, press out excess moisture with paper towels. When this is done, transfer the precipitate to a beaker, and add sufficient alcohol to cover it. (*Caution: Highly inflammable.*) Break up the particles of casein with a glass rod. Let the casein settle and pour off the liquid. Casein does not dissolve in alcohol, but washing it in this way removes impurities which do. Press out the excess alcohol from the precipitate in the beaker with a paper towel. Repeat the alcohol washing. Press out again and set it aside to dry. At the end of the afternoon transfer the casein to a cardboard container.

Filter out the coagulated proteins using the suction funnel. Put a paper filter into the funnel, wet it down with a little water, turn on the aspirator, and connect the suction hose to the flask. Pour in the solution containing the coagulated protein. When the liquid has all passed through, disconnect the suction hose or release pressure by letting air enter. (*Do not* release suction by turning off the aspirator, since this will cause water from the line to back up into the flask.) Pour the filtrate into a beaker. Wash the coagulated protein on the filter pad by pouring alcohol over it and sucking it dry. Do this twice; then remove the material from the filter into a cardboard container with a glass rod or spatula. Label.

Rinse out the beakers and suction flask before using them in subsequent steps.

Evaporate the solution further. Use a very gentle flame and stir constantly with a glass rod, lest the liquid boil out of the beaker. (In heating liquids avoid at any time having your face near the mouth of the beaker or tube so that accidental spurting will not result in injury. And don't forget also to be your neighbor's keeper.) Continue evaporation until a white precipitate of calcium phosphate appears. (The volume of the liquid will probably be about 20 ml.) Cool to room temperature. Filter with suction. Set aside the filtrate. Wash the precipitate three times with small portions (about 5 ml) of cold water. Suck dry. Transfer to another cardboard container.

Evaporate the filtrate over a very gentle flame until it becomes syrupy, or to a volume of about 10 ml. Stir constantly and remove the flame if necessary so that neither excessive foaming nor charring of the sugar occurs. Add this syrup to about 6 ml (about two inches) of acetone in a test tube. (*Caution: Highly inflammable.*) Stopper, and shake to disperse contents. Label the test tube with your name and place it in the refrigerator until next week.

Make a dialysis sac out of cellophane tubing. Obtain about 9 inches of tubing. Don't bend it sharply while it is dry or it will develop leaks. Wet it thoroughly with water, and open it by running water through it. Tie a sturdy knot to seal off one end, and knot it again for good measure. With a pipet introduce 5 ml of milk into the sac. (In using the pipet, hold it between the thumb and middle finger; the index finger over the end acts as a valve.) Carefully tie a knot in the top part of the bag. Put it in a large test tube. Add water to the level of the milk. Cover with a stopper, label, and leave it in the refrigerator.

Digestion of casein

It requires many hours of boiling in strong acid or alkali to hydrolyze a protein molecule. Living systems accomplish the same thing in relatively neutral solution, and at relatively low

temperatures. The difference is that in living organisms such reactions are catalyzed by enzymes. All known enzymes are proteins, and hence possess typical protein properties.

In vertebrates generally, the pancreas secretes into the digestive system a number of enzymes (trypsin, chymotrypsin, etc.) that catalyze the breakdown of proteins into smaller units, called peptides, and individual amino acids. You will be supplied with a dilute pancreatic extract in order to carry out at room temperature the digestion of the casein you have prepared.

Measure about 0.2 gm of casein into a test tube. Add approximately 2 ml of 0.1% pancreatic extract, dissolved in 0.1 *M* phosphate buffer at pH 7.0. Swirl the contents of the test tube to dissolve or suspend the casein. Add a small crystal of thymol to prevent the growth of microorganisms. Label and initial your tube, stopper it, and give it to your instructor to store until the next laboratory period.

A word on molecular structure

Read the material on this topic in Exercise III and make yourselves models of a representative fat, and the sugars glucose and galactose. See what it means to join glucose and galactose together, taking out a molecule of water, to yield lactose. Similarly construct a polypeptide chain from a few generalized amino acids, and see what it means to insert a molecule of water so as to break (hydrolyze) such a chain, the process catalyzed by such protein-hydrolyzing enzymes as are found in pancreatic extracts.

PART 2

(Readings: G. Wald, "The Origin of Life," *Sci. Am.* **191**, No. 2, 45–53, Aug. 1954, Reprint No. 47. P. Doty, "Proteins," *Sci. Am.* **197**, No. 3, 173–184, Sept. 1957, Reprint No. 7. E. O. P. Thompson, "The Insulin Molecule," *Sci. Am.* **192**, No. 5, 36–41, May 1955, Reprint No. 42.)

Shake the tube containing the lactose crystals, and filter with suction. Wash them with two 5-ml portions of acetone. (Acetone is a fire hazard. When you are through, flush it down the drain with plenty of water.) Suck the crystals dry, and transfer them to a cardboard container.

We will now examine some of the specific properties of the substances isolated from milk. Record all observations immediately. First note the colors and textures of these substances. Then carry out the following general tests for protein and sugar on each of the four components.

EXPERIMENTAL PROCEDURE

Prepare two groups of test tubes, with five in each series. Leave one tube of each series empty to serve as a blank. In the other four tubes of each series place a pinch (about the size of a BB) of casein in one, of coagulated protein in another, of lactose in a third and Ca phosphate (or oxalate) in the last.

Biuret test for proteins

Add 3 ml of sodium hydroxide to each of one series of test tubes. Gentle warming of a tube in a water bath will hasten the solution of whatever material it contains, but run the rest at room temperature. Add a few drops of copper sulfate solution. Note any appearance of color. The biuret test is given not only by proteins, but by any substance that contains so-called peptide bonds (—CO—NH—).

Benedict test for sugars

Add 5 ml of Benedict solution to each test tube in the second series. Hold them in the boiling water bath for 2 minutes. Compare the colors. The Benedict test is given by all sugars that contain groups (aldehyde or ketone) that

can reduce blue cupric (Cu^{++}) to red cuprous (Cu^{+}) ions. It is not given by the sugar most familiar to you, sucrose (cane sugar). Why not?

Run the Benedict test with a graded series of sugar concentrations prepared as follows: Label 5 small test tubes, #1 to #5. To a pinch of lactose in test tube #1, add 20 drops of water, and swirl to dissolve. Add 10 drops of water to each of the other 4 test tubes. Now transfer 10 drops of lactose solution from test tube #1 to #2 and mix; then transfer 10 drops from #2 to #3, and so on. Discard 10 drops of solution from tube #5, after mixing. You now have a series of test tubes, each containing half as much sugar as the one before it. Add 3 ml of Benedict solution to each, and heat in the boiling water bath for 2 minutes. Note the colors, and set aside.

Completion of the dialysis experiment

The biuret and Benedict tests can now be used to determine whether protein, sugar, or both have passed out of the dialysis sac prepared last week. Remove the sac from the surrounding solution in the test tube. The latter is called the dialysate. Empty the contents of the sac into a beaker. Test 5 drops of each solution for both sugar and protein. Compare the colors with those of the blanks, prepared above, and with the graded sugar series. Does the dialysate contain sugar? How much sugar, compared with the sac contents? Does it contain protein? How do you explain these observations?

Paper chromatography of amino acids and casein hydrolysate

In this experiment, six known amino acids [alanine, aspartic acid, lysine, proline (an imino acid), histidine and methionine], one unknown amino acid, and your casein hydrolysate are chromatographed on a single sheet of filter paper.

Place a piece of filter paper, 4″ × 5″, on wax paper, and draw a fine line with lead pencil parallel to and 1.5 cm from one long edge, which will be the bottom of your chromatogram. On this line mark pencil dots about 1 cm apart, starting about 2 cm from one edge. These are to indicate the positions for placing your samples; you can label each sample directly on the paper below the line.

The samples are applied to the paper with a fine glass capillary. (The instructor will show you how to make capillaries.) Draw a little solution into a capillary, touch it to the paper at a pencil dot, let dry, and repeat. Each spot should not be more than 3 mm in diameter. Two such superimposed applications should be sufficient with the amino acid solutions, and four with the casein hydrolysate. It will be advantageous to place your unknown amino acid in the middle, between the third and fourth known amino acid. (*Note:* Avoid excess handling of the filter paper, since your hands might contaminate it with amino acids. Touch it only at the edges.) Now roll the sheet into a cylinder, with the short dimension vertical, and tie the edges together with staples so that they do not touch each other, as shown in the diagram in Exercise IV.

Pour about 30 ml of solvent (formic acid: isopropanol : water = 10:70:20) into a quart jar. Line the walls of the jar with a piece of filter paper dipping into the solvent, to act as a wick and help to keep the atmosphere in the jar saturated with solvent. Splash the solvent about. Now insert your cylinder, keeping it away from the walls, close the jar, and let it stand quietly. Wait until the solvent has risen within 0.5 cm from the top of the paper before removing the cylinder and letting it dry. Then dip it into the ninhydrin-acetone reagent, and after the acetone has evaporated, place the paper in the warm oven (80°) for a few minutes. Do not leave it too long or let it overheat! Look each minute, and take it out as soon as you can clearly see the spots. Then immediately outline the spots that you see with pencil. (You can now handle the paper freely but the spots fade in the light.) Which of the amino acids is your unknown?

The ninhydrin test yields purple colors with amino acids and some related substances, and a yellow spot with the imino acid proline.

EQUIPMENT

First Milk Experiment

Per student

$\frac{1}{2}$ pint of milk
1 ft^2 of cloth
9″ of dialysis tubing, $\frac{9}{16}$″ in diameter
large test tube (20 × 150 mm)
test tube (16 × 150 mm)
2 stirring rods
wax pencil
box of matches
4 cardboard containers
No. 0 rubber stopper or cork
No. 4 rubber stopper or cork
bunsen burner
2 400-ml beakers
dropping bottle of 4 *N* hydrochloric acid
spatula
5-ml serological pipet
test-tube rack, with holes 1 in^2
tripod or ring
wire gauze
250-ml beaker

Per 2 students

Buchner funnel, 56 mm in diameter
500-ml suction flask
aspirator

Per 8 students

small bottle of glass beads
2 boxes of paper towels
1 gal 95% alcohol
1 pint of acetone
box of Whatman No. 1 filter paper, 56 mm in diameter
buffer solution (200 ml)
dropping bottle of enzyme solution

Second Milk Experiment

Per student

14 test tubes (16 × 150 mm)
5 small test tubes (13 × 100 mm)
2 6″ medicine droppers
5-ml serological pipet
400-ml beaker
250-ml beaker
spatula
test-tube rack

Per 2 students

Buchner funnel
500-ml suction flask

Per 8 students

acetone
filter paper
10% sodium hydroxide (500 ml)
dropping bottle of 0.5% copper sulfate solution
Benedict solution, 1 liter prepared as directed in Exercise IV

Paper Chromatography Experiment

Per student

soft glass tubing
quart jar
filter paper
stapler
oven
wax paper
beaker (water bath)
2 test tubes, with corks
formic acid
isopropanol
acetone
ninhydrin
alanine (10^{-3} *M*)
aspartic (10^{-3} *M*)
lysine (10^{-3} *M*)
proline (10^{-3} *M*)
histidine (10^{-3} *M*)
methionine (10^{-3} *M*)
pangestin
phosphate buffer (0.1 *M*, pH 7.0)
thymol

EXPONENTS AND LOGARITHMS

APPENDIX D

Number	As power of 10	log
0.001	10^{-3}	-3 or $\bar{3}$
0.01	10^{-2}	-2 or $\bar{2}$
0.1	10^{-1}	-1 or $\bar{1}$
1	10^{0}	0
10	10^{1}	1
100	10^{2}	2
1000	10^{3}	3

Multiplication

$10^3 \times 10^3 = 10^6$ (1000 × 1000 = 1 million),

$\therefore \log 10^3 + \log 10^3 (2 \times \log 10^3) = 3 + 3 = 6$ (i.e., multiplying numbers = adding exponents or logs).

Division

$$10^6 \div 10^2 = 10^4 \left(\frac{1{,}000{,}000}{100} = 10{,}000\right),$$

$\therefore$ in logs: $6 - 2 = 4$ (i.e., dividing numbers = subtracting exponents or logs).

Number	log	Number	log
0	— infinity	6	0.7782
1	0.0000	7	0.8451
2	0.3010	8	0.9031
3	0.4771	9	0.9542
4	0.6021	10	1.0000
5	0.6990		

Make a graph, plotting these numbers against their logs. From this you can interpolate intermediate numbers. From your graph read the logs of 2.35, 9.76, 3.87.

The log of

5.76 = 0.7604,
576 = 2.7604,
576 million = 8.7604,
0.576 = $\bar{1}$.7604,
0.00576 = $\bar{3}$.7604.

The number before the decimal point is called the *characteristic:*

characteristic	43210 $\bar{1}\bar{2}\bar{3}\bar{4}\bar{5}$
number	00000.00000

To multiply decimals:

$$0.003 \times 0.090 = 0.00027$$

logs: $\bar{3}.48 + \bar{2}.95 = \bar{4}.43$

Check with your graph.

An exercise in logarithms: the pH scale

In pure water or in any aqueous solution, whatever its alkalinity or acidity, the product of the concentrations of hydrogen and hydroxyl ions in moles per liter is 10^{-14}:

$$(H^+)(OH^-) = 10^{-14}$$

$$\therefore \log (H^+) + \log (OH^-) = -14$$

or, changing signs,

$$-\log (H^+) - \log (OH^-) = 14$$

$-\log (H^+)$ is called the *pH* (Sörensen). In a neutral solution, $(H^+) = (OH^-) = 10^{-7}$ moles per liter, i.e.,

$$\log (H^+) = -7$$

$$\therefore \text{pH} = 7$$

In an acid solution, pH is less than 7; in an alkaline solution it is more than 7.

pH 0: the solution contains 1 mole (H^+) per liter. Explain.

pH 14: the solution contains 1 mole (OH^-) per liter. Explain.

What is the concentration of (H^+) in a solution of pH 7.35? 9.73?

What is the approximate pH of a solution containing: 0.03 moles per liter of hydrochloric acid? 0.0007 moles per liter of potassium hydroxide?

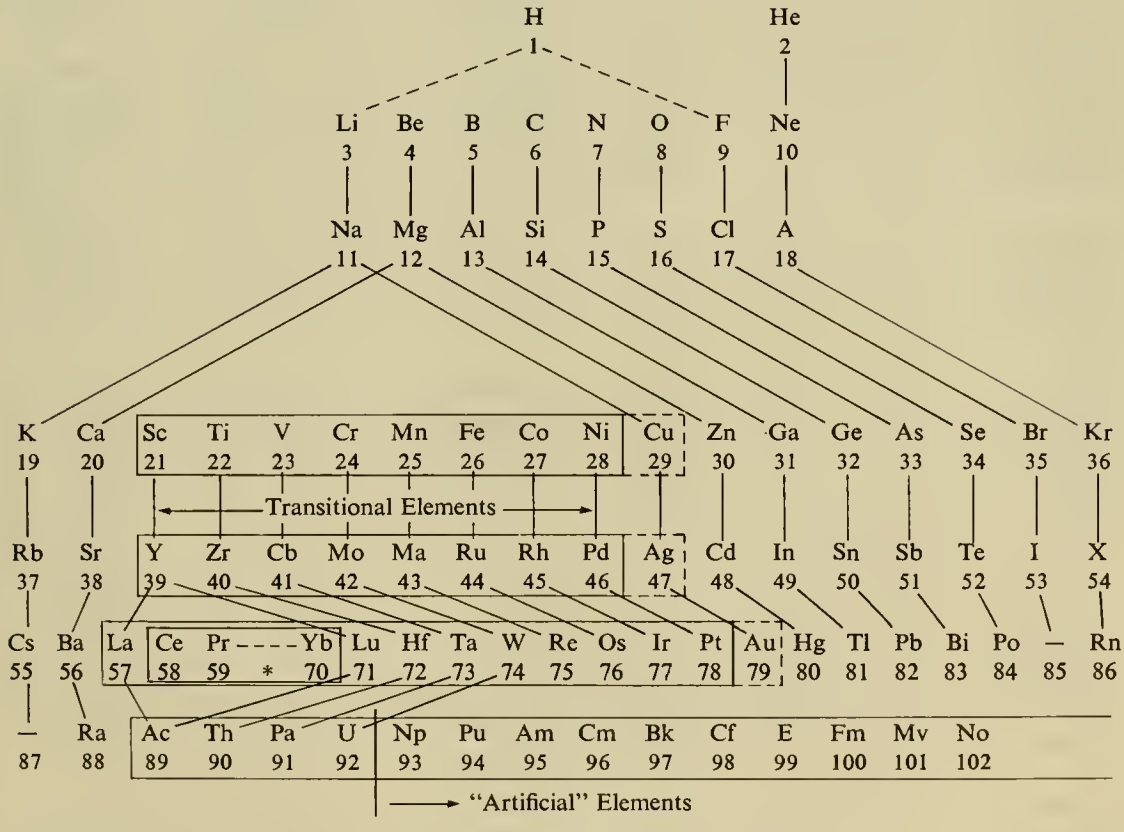
THE PERIODIC SYSTEM OF THE ELEMENTS

(Niels Bohr's Arrangement)

APPENDIX E

ELECTRONIC CONFIGURATIONS OF THE INERT GASES

Atom and atomic number	*Electrons in quantum groups* 1st	2nd	3rd	4th	5th	6th
Helium (2)	2					
Neon (10)	2	8				
Argon (18)	2	8	8			
Krypton (36)	2	8	18	8		
Xenon (54)	2	8	18	18	8	
Radon (86)	2	8	18	32	18	8



*Rare earths: Nd 60 Pm 61 Sm 62 Eu 63 Gd 64 Tb 65 Dy 66 Ho 67 Er 68 Tm 69

TABLE OF ATOMIC WEIGHTS

APPENDIX F

Element	Symbol	At. no. Z	At. wt. (chem. scale)
Actinium	Ac	89	(227)
Aluminum	Al	13	26.98
Americium	Am	95	(243)
Antimony	Sb	51	121.76
Argon	Ar	18	39.944
Arsenic	As	33	74.91
Astatine	At	84	(210)
Barium	Ba	56	137.36
Berkelium	Bk	97	(247)
Beryllium	Be	4	9.013
Bismuth	Bi	83	(209)
Boron	B	5	10.82
Bromine	Br	35	79.916
Cadmium	Cd	48	112.41
Calcium	Ca	20	40.08
Californium	Cf	98	(251)
Carbon	C	6	12.011
Cerium	Ce	58	140.13
Cesium	Cs	55	132.91
Chlorine	Cl	17	35.457
Chromium	Cr	24	52.01
Cobalt	Co	27	58.94
Copper	Cu	29	63.54
Curium	Cm	96	(247)
Dysprosium	Dy	66	162.51
Einsteinium	Es	99	(254)
Emanation	Em	86	(222)(Rn)
Erbium	Er	68	167.27
Europium	Eu	63	152.0
Fermium	Fm	100	(253)
Fluorine	F	9	19.00
Francium	Fr	87	(223)
Gadolinium	Gd	64	157.26
Gallium	Ga	31	69.72
Germanium	Ge	32	72.60
Gold	Au	79	197.0
Hafnium	Hf	72	178.50
Helium	He	2	4.003
Holmium	Ho	67	164.94
Hydrogen	H	1	1.0080
Indium	In	49	114.82
Iodine	I	53	126.91
Iridium	Ir	77	192.2
Iron	Fe	26	55.85
Krypton	Kr	36	83.80
Lanthanum	La	57	138.92
Lead	Pb	82	207.21
Lithium	Li	3	6.940
Lutetium	Lu	71	174.99
Magnesium	Mg	12	24.32
Manganese	Mn	25	54.94
Mendelevium	Md	101	(256)
Mercury	Hg	80	200.61
Molybdenum	Mo	42	95.95
Neodymium	Nd	60	144.27
Neon	Ne	10	20.183
Neptunium	Np	93	(237)
Nickel	Ni	28	58.71
Niobium or (Columbium)(Cb)	Nb	41	92.91
Nitrogen	N	7	14.008
Nobelium	No	102	(253)
Osmium	Os	76	190.2
Oxygen	O	8	16.0000 (standard)

(*cont.*)

TABLE OF ATOMIC WEIGHTS (*cont.*)

Element	Symbol	At. no. Z	At. wt. (chem. scale)	Element	Symbol	At. no. Z	At. wt. (chem. scale)
Palladium	Pd	46	106.4	Tantalum	Ta	73	180.95
Phosphorus	P	15	30.975	Technetium	Tc	43	(98)
Platinum	Pt	78	195.09	Tellurium	Te	52	127.61
Plutonium	Pu	94	(244)	Terbium	Tb	65	158.93
Polonium	Po	84	(210)	Thallium	Tl	81	204.39
Potassium	K	19	39.100	Thorium	Th	90	(232)
Praseodymium	Pr	59	140.92	Thulium	Tm	69	168.94
Promethium	Pm	61	(145)	Tin	Sn	50	118.70
Protactinium	Pa	91	(231)	Titanium	Ti	22	47.90
				Tungsten (Wolfram)	W	74	183.86
Radium	Ra	88	(226)				
Rhenium	Re	75	186.22	Uranium	U	92	(238)
Rhodium	Rh	45	102.91				
Rubidium	Rb	37	85.48				
Ruthenium	Ru	44	101.10	Vanadium	V	23	50.95
Samarium	Sm	62	150.35	Xenon	Xe	54	131.30
Scandium	Sc	21	44.96				
Selenium	Se	34	78.96				
Silicon	Si	14	28.09	Ytterbium	Yb	70	173.04
Silver	Ag	47	107.88	Yttrium	Y	39	88.92
Sodium	Na	11	22.991				
Strontium	Sr	38	87.63	Zinc	Zn	30	65.38
Sulfur	S	16	32.066	Zirconium	Zr	40	91.22

BIBLIOGRAPHY

ARTICLES FROM *SCIENTIFIC AMERICAN*

INTRODUCTION

WALD, G., *Innovation in Biology*, **199**, No. 3, 100–113, Sept. 1958.
WALD, G., *The Origin of Life*, **191**, No. 2, 45–53, Aug. 1954.

THE COSMOLOGICAL SETTING

GAMOW, G., *The Evolutionary Universe*, **195**, No. 3, 136–154, Sept. 1956.
HOYLE, F., *The Steady-State Universe*, **195**, No. 3, 157–166, Sept. 1956.
SANDAGE, A. R., *The Red-Shift*, **195**, No. 3, 170–182, Sept. 1956.

ELEMENTARY PARTICLES

BURBIDGE, G., and HOYLE, F., *Anti-Matter*, **198**, No. 4, 34–39, April 1958.
GAMOW, G., *The Principle of Uncertainty*, **198**, No. 1, 51–57, Jan. 1958.
GELL-MANN, M., and ROSENBAUM, E. P., *Elementary Particles*, **197**, No. 1, 72–88, July 1957.
MORRISON, P., and MORRISON, E., *The Neutron*, **185**, No. 4, 44–53, Oct. 1951.

ATOMS

BROWN, H., *The Age of the Solar System*, **196**, No. 4, 80–94, April 1957.
DARROW, K. K., *The Quantum Theory*, **186**, No. 3, 47–54, March 1952.
FOWLER, W. A., *The Origin of the Elements*, **195**, No. 3, 82–91, Sept. 1956.
GAMOW, G., *The Exclusion Principle*, **201**, No. 1, 74–86, July 1959.
PEIERLS, R. E., *The Atomic Nucleus*, **200**, No. 1, 75–82, Jan. 1959.
PERLMAN, I., and SEABORG, G. T., *The Synthetic Elements, I*, **182**, No. 4, 38–47, April 1950.

MOLECULES

BUSWELL, A. H., and RODEBUSH, W. H., *Water*, **194**, No. 4, 76–89, April 1956.
ROBERTS, J. D., *Organic Chemical Reactions*, **197**, No. 5, 117–126, Nov. 1957.
WANNIER, G. H., *The Nature of Solids*, **187**, No. 6, 39–48, Dec. 1952.

PHYSICAL METHODS FOR THE STUDY OF MOLECULES

GRAY, G. W., *Electrophoresis*, **185**, No. 6, 45–53, Dec. 1951.
GRAY, G. W., *The Ultracentrifuge*, **184**, No. 6, 42–51, June 1951.
KAMEN, M. D., *Tracers*, **180**, No. 2, 30–41, Feb. 1949.
STEIN, W. H., and MOORE, S., *Chromatography*, **184**, No. 3, 35–41, March 1951.

MOLECULES OF LIVING ORGANISMS

ABELSON, P. H., *Paleobiochemistry* (10), **195,** No. 1, 83–92, July 1956.

CRICK, F. H. C., *The Structure of the Hereditary Material*, **191,** No. 4, 54–61, Oct. 1954.

DOTY, P., *Proteins*, **197,** No. 3, 173–184, Sept. 1957.

FIESER, L. F., *Steroids*, **192,** No. 1, 52–60, Jan. 1955.

PAULING, L., COREY, R. B., and HAYWARD, R., *The Structure of Protein Molecules*, **191,** No. 1, 51–59, July 1954.

STEIN, W. H., and MOORE, S., *The Structure of Proteins*, **204,** No. 2, 81–92, Feb. 1961.

THOMPSON, E. O. P., *The Insulin Molecule*, **192,** No. 5, 36–41, May 1955.

ENERGY TRANSFORMATIONS IN CELLS

ARNON, D. I., *The Role of Light in Photosynthesis*, **203,** No. 5, 104–118, Nov. 1960.

BASSHAM, J. A., *The Path of Carbon in Photosynthesis*, **206,** No. 6, 88–100, June 1962.

LEHNINGER, A., *Energy Transformation in the Cell*, **202,** No. 5, 102–114, May 1960. *How Cells Transform Energy*, **205,** No. 3, 62–73, Sept. 1961.

RABINOWITCH, E. I., *Photosynthesis*, **179,** No. 2, 24–34, Aug. 1948.

WALD, G., *Life and Light*, **201,** No. 4, 92–108, Sept. 1959.

GENES, VIRUSES, GENE ACTION

ALLFREY, V. G., and MIRSKY, A. E., *How Cells Make Molecules*, **205,** No. 3, 74–82, Sept. 1961.

BEADLE, G., *The Genes of Men and Molds*, **179,** No. 3, 30–38, Sept. 1948.

BENZER, S., *The Fine Structure of the Gene*, **206,** No. 1, 70–84, Jan. 1962.

BURNET, F. M., *Viruses*, **184,** No. 5, 43–51, May 1951.

FRAENKEL-CONRAT, H., *Rebuilding a Virus*, **194,** No. 6, 42–47, June 1956.

HOAGLAND, M. B., *Nucleic Acids and Proteins*, **201,** No. 6, 55–61, Dec. 1959.

HOROWITZ, N. H., *The Gene*, **195,** No. 4, 78–90, Oct. 1956.

HOTCHKISS, R. D., and WEISS, E., *Transformed Bacteria*, **195,** No. 5, 48–53, Nov. 1956.

HURWITZ, J., and FURTH, J. J., *Messenger R.N.A.*, **206,** No. 2, 41–49, Feb. 1962.

INGRAM, V. M., *How Do Genes Act?*, **198,** No. 1, 68–74, Jan. 1958.

MIRSKY, A. E., *The Chemistry of Heredity*, **188,** No. 2, 47–57, Feb. 1953.

STENT, G. S., *The Multiplication of Bacterial Viruses*, **188,** No. 5, 36–39, May 1953.

WOLLMAN, E. L., and JACOB, F., *Sexuality in Bacteria*, **195,** No. 1, 109–118, July 1956.

ZINDER, N. D., *Transduction in Bacteria*, **199,** No. 5, 38–42, Nov. 1958.

THE CELL AND HEREDITY

BRACHET, J., *The Living Cell*, **205,** No. 3, 50–62, Sept. 1961.

DOBZHANSKY, T., *The Genetic Basis of Evolution*, **182,** No. 1, 32–40, Jan. 1950.

HOLTER, H., *How Things Get into Cells*, **205,** No. 3, 167–180, Sept. 1961.

MAZIA, D., *Cell Division*, **189,** No. 2, 53–63, Aug. 1953. *How Cells Divide*, **205,** No. 3, 100–120, Sept. 1961.

MOROWITZ, H. J., and TOURTELLOTTE, M. E., **206,** No. 3, 117–126, March 1962.

MULLER, H. J., *Radiation and Human Mutation*, **193,** No. 5, 58–68, Nov. 1955.

ROBERTSON, J. D., *The Membrane of the Living Cell*, **206**, No. 4, 65–72, April 1962.

SONNEBORN, T. M., *Partner of the Genes*, **183**, No. 5, 30–39, Nov. 1950.

TAYLOR, J. H., *The Duplication of Chromosomes*, **198**, No. 6, 36–42, June 1958.

EMBRYONIC DEVELOPMENT

BUTLER, W. L., and DOWNS, R. J., *Light and Plant Development*, **203**, No. 6, 56–63, Dec. 1960.

EBERT, J. D., *The First Heartbeats*, **200**, No. 3, 87–96, March 1959.

FISCHBERG, M., and BLACKLER, A. W., *How Cells Specialize*, **205**, No. 4, 124–140, Sept. 1961.

GRANT, V., *The Fertilization of Flowers*, **184**, No. 6, 52–56, June 1951.

GRAY, G. W., "*The Organizer*," **197**, No. 5, 79–88, Nov. 1957.

MOSCONA, A. A., *How Cells Associate*, **205**, No. 4, 142–162, Sept. 1961.

SINGER, M., *Regeneration of Body Parts*, **199**, No. 4, 79–88, Oct. 1958.

TYLER, A., *Fertilization and Antibodies*, **190**, No. 6, 70–75, June 1954.

WADDINGTON, C. H., *How Do Cells Differentiate?*, **189**, No. 3, 108–116, Sept. 1953.

WIGGLESWORTH, V. S., *Metamorphosis and Differentiation*, **200**, No. 2, 100–110, Feb. 1959.

WILLIAMS, C. M., *The Metamorphosis of Insects*, **182**, No. 4, 24–28, April 1950.

ORGANS: STRUCTURE AND FUNCTION

HAYASHI, T., *How Cells Move*, **205**, No. 3, 184–202, Sept. 1961.

HUXLEY, H. E., *The Contraction of Muscle*, **199**, No. 5, 66–82, Nov. 1958.

KATZ, B., *The Nerve Impulse*, **187**, No. 5, 55–64, Nov. 1952. *How Cells Communicate*, **205**, No. 3, 209–220, Sept. 1961.

KEYNES, R. D., *The Nerve Impulse and the Squid*, **199**, No. 6, 83–90, Dec. 1958.

LOEWENSTEIN, W. R., *Biological Transducers*, **203**, No. 2, 98–108, Aug. 1960.

MILLER, W. H., RATLIFF, F., and HARTLINE, H. K., *How Cells Receive Stimuli*, **205**, No. 3, 222–238, Sept. 1961.

SMITH, H. W., *The Kidney*, **188**, No. 1, 40–48, Jan. 1953.

VON BEKESY, G., *The Ear*, **197**, No. 2, 66–78, Aug. 1957.

WALD, G., *Eye and Camera*, **183**, No. 2, 32–40, Aug. 1950.

WIGGERS, C. J., *The Heart*, **196**, No. 5, 74–87, May 1957.

NERVOUS INTEGRATION

ECCLES, J. C., *The Physiology of the Imagination*, **199**, No. 3, 135–142, Sept. 1958.

FRENCH, J. D., *The Reticular Formation*, **196**, No. 5, 54–60, May 1957.

GRAY, G. W., *The Great Ravelled Knot*, **179**, No. 4, 26–39, Oct. 1948.

OLDS, J., *Pleasure Centers in the Brain*, **195**, No. 4, 105–116, Oct. 1956.

SNIDER, R. S., *The Cerebellum*, **199**, No. 2, 84–90, Aug. 1958.

SPERRY, R. W., *The Growth of Nerve Circuits*, **201**, No. 5, 68–75, Nov. 1959.

WALTER, W. G., *The Electrical Activity of the Brain*, **190**, No. 6, 54–63, June 1954.

BEHAVIOR AND SOCIAL INTEGRATION

TINBERGEN, N., *The Evolution of Behavior in Gulls*, **203**, No. 6, 118–130, Dec. 1960.

BOOKS

PHYSICS

BONNER, F. T., and PHILLIPS, M., *Principles of Physical Science.* Addison-Wesley, 1957.

FEATHER, N., *Mass, Length and Time.* Edinburgh Univ. Press, 1959.

HECHT, SELIG, *Explaining the Atom.* Viking Press, 2nd ed., 1954.

HOLTON, G., and ROLLER, D. H. D., *Foundations of Modern Physical Science.* Addison-Wesley, 1958.

MCCUE, J. J. G., *The World of Atoms.* Ronald Press, 1956.

ROGERS, E. M., *Physics for the Inquiring Mind*, Princeton Univ. Press, 1960.

CHEMISTRY

BROWN, G. I., *Simple Guide to Modern Valency Theory.* Longmans, Green, 1954.

BROWN, G. I., *Electronic Theories of Organic Chemistry.* Longmans, Green, 1958.

CRAM, D. J., and HAMMOND, G. S., *Organic Chemistry.* McGraw-Hill, 1959.

Handbook of Chemistry and Physics. Chemical Rubber Pub. Co., Cleveland, Ohio.

HITCHCOCK, D. I., *Physical Chemistry.* Little, Brown, 4th ed., 1953.

LESSING, L. P., *Understanding Chemistry.* Interscience Pub. Co., 1959.

PAULING, L., *College Chemistry.* Freeman, 1957.

PAULING, L., *General Chemistry.* Freeman, 1953.

SEABORG, G. T., and VALENS, E. G., *Elements of the Universe.* Dutton, 1958.

SPEAKMAN, J. C., *Introduction to the Electronic Theory of Valency.* London: Arnold, 1955.

STOTT, R. W., *Electronic Theory and Chemical Reactions.* Longmans, Green, 1958.

BIOLOGY

ADRIAN, E. D., *Physical Background of Perception.* Oxford Univ. Press, 1947.

ADRIAN, E. D., *Mechanism of Nervous Action.* Univ. of Penn. Press, 1932.

BEST, C. H., and TAYLOR, N. B., *The Livig Body.* Henry Holt, 4th ed., 1958.

BONNER, J. T., and GALSTON, A. W., *Principles of Plant Physiology.* Freeman, 1952.

BRAZIER, M. A. B., *Electrical Activity of the Nervous System.* Macmillan, 1951.

BUCHSBAUM, RALPH M., *Animals without Backbones.* Univ. of Chicago Press, rev. ed., 1948.

CANNON, W. B., *Wisdom of the Body.* Norton, 1939.

CARTER, G. S., *General Zoology of the Invertebrates.* Sidgwick and Jackson, 1951.

CONANT, J. B., ed., *Case Histories in Science.* No. 7, "Pasteur's and Tyndall's Spontaneous Generation." Harvard Univ. Press, 1953.

ELLIOTT, A. M., and RAY, C., JR., *Biology.* Appleton-Century-Crofts, 1960.

EVLENBERG-WIENER, *Fearfully and Wonderfully Made.* Macmillan, 1938.

GABRIEL, M., and FOGEL, S., eds., *Great Experiments in Biology.* Prentice-Hall, 1955.

GARROD, A. E., *Inborn Errors of Metobolism.* London: Froude, Hodder and Stoughton, 2nd ed., 1923.

GRAY, JAMES, *How Animals Move.* Cambridge Univ. Press, 1960.

GRIFFIN, D. R., *Echoes of Bats and Men.* Anchor Books, 1959.

GRIFFIN, D. R., *Listening in the Dark.* Yale Univ. Press, 1958.

HARVEY, W., *Motion of the Heart and Blood.* Translated by R. Willis. Everyman.

LOCY, W. A., *The Story of Biology.* Garden City, 1925.

LURIA, S. E., *General Virology*. Wiley, 1953.
MARSLAND, DOUGLAS, *Principles of Modern Biology*. Henry Holt, 3rd ed., 1957.
MEDAWAR, P., *Uniqueness of the Individual*. Methuen, 1957.
OGINSKY, E. L., and UMBREIT, W. W., *Introduction to Bacterial Physiology*. Freeman, 1959.
PIRENNE, M. H., *Vision and the Eye*. Chapman and Hall, 1948.
PROSSER, C. L., ed., *Comparative Animal Physiology*. Saunders, 1950.
ROMER, A. S., *The Vertebrate Story*. Univ. of Chicago Press, 1959.
ROMER, A. S., *Man and the Vertebrates*. Pelican Books.
SCHMIDT-NIELSEN, K., *Animal Physiology*. Prentice-Hall, 1960.
SHERRINGTON, C. S., *Integrative Action of the Nervous System*. Yale Univ. Press, 1952.
SINNOTT, E. W., DUNN, L. C., and DOBZHANSKY, TH., *Principles of Genetics*. McGraw-Hill, 5th ed., 1958.
SRB, A. M., and OWEN, R. D., *Genetics*. Freeman, 1952.
STANIER, R. Y., DOUDEROFF, M., and ADELBERG, E. A., *The Microbial World*. Prentice-Hall, 1957.
SUSSMAN, MAURICE, *Animal Growth and Development*. Prentice-Hall, 1960.
SWANSON, C. P., *The Cell*. Prentice-Hall, 1960.
THIMANN, K. V., *The Life of Bacteria*. Macmillan, 1955.
TINBERGEN, N., *Social Behavior in Animals*. Wiley, 1953.
VILLEE, C. E., *Biology*. Saunders, 3rd ed., 1957.
VON FRISCH, KARL, *The Dancing Bees*. Harcourt, 1955.
WADDINGTON, C. H., *How Animals Develop*. Norton, 1936.
WALTER, W. G., *The Living Brain*. Norton, 1953.
WINCHESTER, A. M., *Genetics*. Houghton Mifflin, 1951.
YOUNG, J. Z., *The Life of Mammals*. Oxford Univ. Press, 1957.
YOUNG, J. Z., *The Life of Vertebrates*. Oxford Univ. Press, 1950.

GENERAL PERSPECTIVES

ANFINSEN, C. B., *The Molecular Basis of Evolution*. Wiley, 1959.
BLUM, H. P., *Time's Arrow and Evolution*. Princeton Univ. Press, 1951.
HENDERSON, L. J., *Fitness of the Environment*. Beacon Press, 1958.
OPARIN, A. I., *The Origin of Life on the Earth*. Oliver and Boyd, 1957.
RUSH, J. H., *The Dawn of Life*. Hanover House, 1957.
SCHRÖDINGER, E., *What is Life?* Cambridge Univ. Press, 1944.
SIMPSON, G. G., *The Meaning of Evolution*. Yale Univ. Press, 1950.
SMITH, H. W., *From Fish to Philosopher*. Little, Brown, 1953.
SNOW, C. P., *Two Cultures* (Oxford).

BIOCHEMISTRY

BALDWIN, ERNEST, *Dynamic Aspects of Biochemistry*. Cambridge Univ. Press, 3rd ed., 1957.
BALDWIN, ERNEST, *Introduction to Comparative Biochemistry*. Cambridge Univ. Press, 3rd ed., 1948.
BOREK, E., *Man, The Chemical Machine*. Columbia Univ. Press, 1953.
BUTLER, J. A. V., *Inside the Living Cell*. Basic Books, 1959.
HARRISON, K., *A Guide-Book to Biochemistry*. Cambridge Univ. Press, 1959.
HARROW, B., and MAZUR, A., *Textbook of Biochemistry*. Saunders, 1958.

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ADLER, IRVING, *How Life Began*. Signet.

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CROMPTON, JOHN, *The Life of the Spider*. Mentor.

DARWIN, CHARLES, *The Origin of Species* (Mentor); *The Voyage of the Beagle* (Bantam).

DAVIS, HELEN MILES, *The Chemical Elements*. Ballantine Books.

DE KRUIF, PAUL, *Microbe Hunters*. Cardinal.

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HOYLE, FRED, *Frontiers of Astronomy* (Mentor); *The Nature of the Universe* (Mentor).

HUTTON, KENNETH, *Chemistry, The Conquest of Materials*. Penguin Books.

HUXLEY, JULIAN, *Evolution in Action*. Mentor.

JEANS, JAMES, *The Growth of Physical Science*. Premier.

JONES, H. SPENCER, *Life on Other Worlds*. Mentor.

LESSING, LAWRENCE P., *Understanding Chemistry*. Mentor.

MALTHUS, THOMAS, JULIAN HUXLEY, and FREDERICK OSBORN, *Three Essays on Population*. Mentor.

OPIK, ERNST J., *The Oscillating Universe*. Mentor.

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RAPPORT, SAMUEL, and HELEN WRIGHT, editors, *The Crust of the Earth*. Mentor.

SANDERSON, IVAN T., *How to Know the American Mammals*. Signet.

SHAPLEY, HARLOW, *Of Stars and Men* (Washington Square Press); *Stars in the Making* (Pocket Books).

SIMPSON, GEORGE GAYLORD, *The Meaning of Evolution*. Mentor.

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WHITEHEAD, ALFRED NORTH, *Science and the Modern World*. Mentor.

FICTION

CAPEK, KAREL, *War with the Newts*. Bantam.

HOYLE, FRED, *The Black Cloud*. Signet.

LEWIS, SINCLAIR, *Arrowsmith*. Harcourt.

SNOW, C. P., *The Affair* (Signet); *The Search* (Signet).